Synopsis of the thesis on

Development of nano diagnostic platform for potato virus detection

To be submitted to

Institute of Advanced Research

For the degree of

Doctor of Philosophy in Biotechnology

by

Nidhi Verma

UIAR-606

Under the Guidance of

Dr. Alok Pandya

and co-guidance of

Dr. Budhi Sagar Tiwari

Department of Biotechnology and Bioengineering

Institute of Advanced Research

Abstract

Potato, as a predominant non-grain food crop globally, is under threat from plant viruses, jeopardizing food supply sustainability. Early and accurate detection of potato plant viruses is vital for effective monitoring and control. Paper-based biosensors offer a compelling solution with their simplicity, affordability, and portability, enabling rapid on-site detection of biomarkers and pollutants, especially in resource-limited settings. To maximize the potential of paper-based sensors, we developed a vertical flow-based immunodevice (VFID) specifically designed for detecting Potato virus Y (PVY) in potato plants. The VFID assay exhibited outstanding performance, demonstrating excellent accuracy, sensitivity, and reliability when validated with potato leaf samples. Furthermore, we explored the use of electrochemical sensors employing shape-dependent nanoparticles such as gold nanoparticles and reduced graphene oxide for enhanced sensitivity in potato virus detection. Our findings revealed that electrochemical immunoelectrodes based on spherical AuNP and rGO nanosheets achieved excellent limit of detection at concentrations of 0.96 x 10^{-12} M and 0.065 x 10^{-12} M, respectively. To leverage the advantages of both technologies, we developed a paper disc interfaced immunodevice capable of detecting plant biomarkers, specifically targeting Potato virus X. Remarkably, this device exhibited a detection limit of 0.92 nM and showcased versatility by effectively detecting other biomarkers, including potato aucuba mosaic virus in plant diseases and C-reactive protein in human diseases. These innovative immunodevices hold significant promise for protecting potato crops and ensuring global food security.

Keywords: Sensor ; Potato Virus ; Electrochemical; Point of care ; Microfluidic ; Nanodiagnostic ; Detection

Contents

Abstract **iii**

Content **iv**

1 Introduction **1**
1.1 The correlation between population and food requirement 1
1.2 Current scenario of crop loss 2
1.3 Review of literature 3
1.3.1 Potato as a crop of imminence around the globe 3
1.3.2 Potato virus - impacting agriculture 3
1.3.3 Potato viruses around various continents 4
1.4 Detection tools for plant pathogen detection 5
1.5 Detection of plant diseases using portable sensors 7
1.5.1 Microfluidic-based portable sensor for plant pathogen detection 7
1.5.2 Multiplexed point-of-care diagnostic platform for plant pathogens 9
1.6 Research gap 10
1.7 Scope: Unmet Need 11

2 Development of Vertical flow immunoassay for detection of PVY **13**
2.1 Introduction 13
2.2 Assay Mechanism and Interpretation of VFID 14
2.3 Results and Discussion 15
2.4 Conclusion 18

3 Development of Electrochemical immunosensor for detection of PAMV **19**
3.1 Immobilization strategy 20
3.1.1 Quantitative capability of PAMV immunosensor based on active antibody 21
3.2 Percentage availability of active antibody 21
3.3 Sensitivity of fabricated immunosensor 22

3.4 Conclusion 24

4 Development of electro-chemical immuno-sensor for diverse bio-markers 25

4.1 Introduction 25
4.2 Immunodevice fabrication and detection mechanism 27
4.3 Analytical performance of the immunodevice 28
4.4 Validation of the immunodevice performance in real samples 28
4.5 Extension of the immunodevice platform for the detection of diverse biomarkers 32
4.6 Conclusion 34

List of Publications 34

References 38

Chapter 1

Introduction

1.1 The correlation between population and food requirement

The increasing population across the globe and its consequent rise in food demand are putting immense pressure on agriculture and natural resources. However, the current food systems are not capable of providing sufficient nutritious food to the world's population in an environmentally sustainable way[1]. Currently, there are 821 million undernourished individuals and 1.2 billion who are either overweight or obese, highlighting imbalances in the global food system[2]. Meanwhile, food production, processing, and waste are also posing unsustainable pressure on the environment. The Food and Agriculture Organization (FAO) 2009 report states that by 2050, the world population of 9.7 billion will demand 70% more food than the present consumption level[3].To ensure the nutritional needs of an expanding population are met sustainably, substantial improvements to the global food system are imperative. To ensure the nutritional needs of an expanding population are met sustainably, substantial improvements to the global food system are imperative. These enhancements should encompass advancements that facilitate the well-being of farmers, offer consumers access to nutritious products, and mitigate adverse environmental repercussions. Based on the Global Hunger Index (GHI) report by Von Grebmer et al. (2017), significant strides have been made in reducing hunger in the developing world. The GHI evaluates countries on a scale of 0-100, with 0 indicating no hunger and 100 representing the worst score. The 2017 GHI score for the developing world was 21.8, a 27% decrease from the 2000 score of 29.9[4]. However, there are still substantial disparities in hunger at various levels, and progress has not been consistent.

1.2 Current scenario of crop loss

Globally, plant diseases have caused significant crop losses, hindering efforts to improve crop productivity and overall production. Despite advancements in plant breeding and disease management techniques, crop losses due to diseases remain a major obstacle for agriculture worldwide, including in developed nations. In recent years, there have only been marginal increases in the yield of major food crops, and with projections of world food deficits and expanding populations, the international scientific community has renewed its interest in researching the relationship between disease and loss. Accurately quantifying crop loss is essential for sound plant protection programs, which are crucial for problem-solving in agriculture. The development of rational or optimal strategies for managing diseases by farmers or national and international agencies cannot be expected without conducting appropriate assessments. Based on the available data from Europe, Africa, Australia, and Asia, it has been found that the most commonly cultivated crops around the world include corn, potato, rice, soybean, and wheat. This information is presented in **Figure 1.1** that specifically highlights these crops by encircling them with black. It is important to note that the data is cumulative, meaning it has been collected over a period of time and from multiple sources. These crops are of great significance to global agriculture as they play a major role in providing food, income, and other resources to many populations around the world. Understanding the most commonly cultivated crops can help inform agricultural policies, resource allocation, and research efforts aimed at improving crop yields and addressing food security concerns as shown in **Figure 1.1**. The figure not only highlights the most cultivated crops but also provides information on the top pathogens that affect different continents. The data suggests that *Tomato yellow leaf curl virus, Tobacco mosaic virus, Tomato spotted wilt virus, Ralstonia solanacearum, and Agrobacterium tumefaciens* are some of the most significant pathogens worldwide, contributing to significant economic and crop losses. These pathogens have a wide distribution and affect a variety of crops, including important food crops such as tomatoes, tobacco, and many others. Understanding the impact of these pathogens on global crop production is crucial for developing effective strategies to mitigate their effects, minimize losses, and improve crop yields.

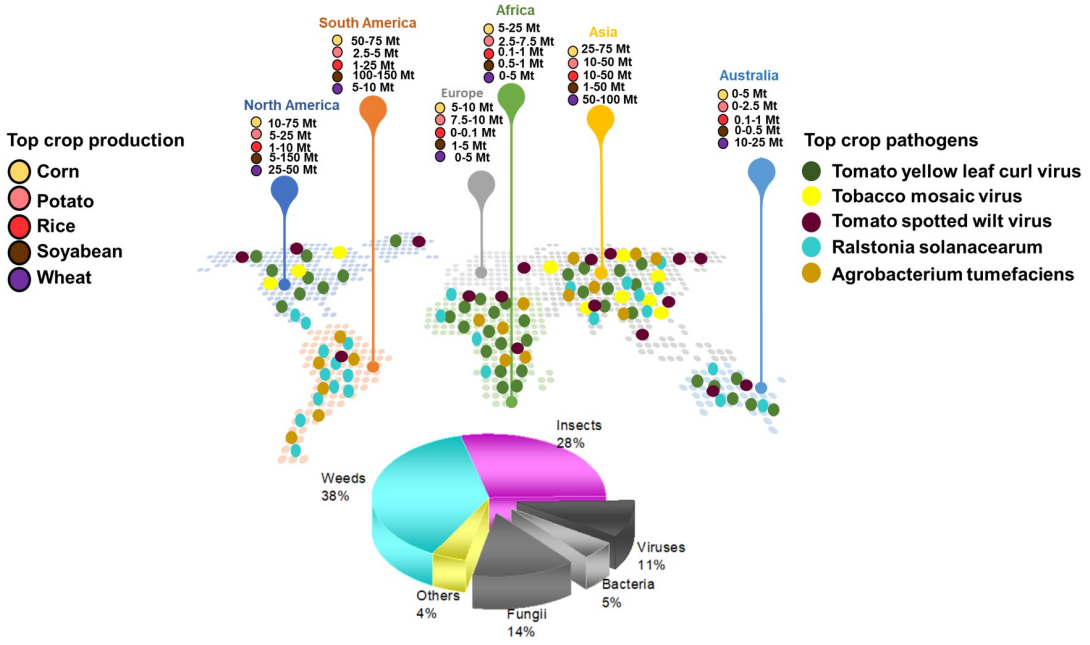


Figure 1.1: Global burden of plant pathogens[5]

1.3 Review of literature

1.3.1 Potato as a crop of imminence around the globe

The potato (*Solanum tuberosum L.*) is a crucial crop for human consumption, ranking third after rice and wheat globally. However, India holds the second position in potato production after China, with average productivity of one million hectares and tonnes per hectare in 2022[6] (FAOSTAT 2017). Potato is an important staple crop that has been a part of the human diet for thousands of years. It was first domesticated in the Andean region of South America and then spread throughout the world. Today, potatoes are one of the most widely consumed crops in the world and are an important source of nutrition for millions of people. In 2021, the FAO reported that global potato production reached an impressive 376 million tonnes. China and India were the two largest potato-producing countries, with China producing 94 million tonnes and India producing 54 million tonnes[7].

1.3.2 Potato virus - impacting agriculture

Over the past few decades, there has been a significant increase in potato production worldwide compared to other major staple crops. This increase has been mainly seen in developing countries located in tropical and subtropical regions. One of the primary

reasons for the popularity of potato cultivation among farmers is its ability to produce high amounts of digestible energy per unit area for both home consumption and as a cash crop.

Looking ahead, the development of new heat-tolerant and early-maturing potato cultivars is expected to further expand potato production into warmer areas of the tropics. However, rising temperatures could also lead to an increase in virus vectors, which are insects that transmit diseases, and the incidence of virus epidemics.

Unfortunately, virus-tested seed systems are weak or entirely absent in many developing countries, making potato virus diseases a significant concern. In fact, these diseases are estimated to account for 50% or more of the potential total yield lost. Therefore, it is crucial to address the issue of potato virus diseases to ensure sustainable potato production and food security in the developing world[8]. With the world's population continually growing, feeding the masses will become increasingly challenging, and increasing agricultural productivity is imperative. Unfortunately, plant pathogens such as fungi, bacteria, nematodes, and viruses frequently cause diseases that significantly impact agricultural productivity.

The rate of infection varies significantly based on secondary infections with other potato viruses. For example, a loss of 5-15% in tuber yield is reported if the plant is infected with Potato virus X (PVX) and Potato virus S (PVS), while 15-30% loss is reported for secondary infection of Potato virus Y strain N (PVY^n) and 40-70% due to infection of Potato leaf roll virus (PLRV). Additionally, Potato spindle tuber viroid (PSTVd) is prevalent in Europe and North America and can significantly reduce yields (16-64%) depending on the viroid strain and potato variety and warmer weather[9]. Severe strains of PVY and PLRV can reduce production by up to 80%, while mild viruses such as PVX, PVS, and Potato virus M (PVM) can reduce yield by up to 30%[10]. In India, the whitefly-transmitted begomovirus ToLCNDV-potato, causing apical leaf curl disease in potatoes, has become a significant concern, with up to 40% infections reported in traditional cultivars, resulting in significant losses[11,12].

1.3.3 Potato viruses around various continents

The introduction of potato crops to Europe prior to 1570 resulted in their degeneration or "running off," which was initially attributed to changes in cultivars arising from continuous vegetative propagation. It was not until the 19th century that it was discovered that degeneration was actually caused by diseases, particularly those caused by viruses[13]. Subsequently, substantial efforts have been undertaken to control virus diseases in potato crops by eliminating them from clonal and seed-propagated stocks, which has been reliant on the accurate identification of causal viruses and the develop-

ment of low-cost, sensitive virus detection technology[14]. In addition to a few phytoplasma organisms and the potato spindle tuber viroid (PSTVd), more than 35 viruses are known to affect potato crops, with potato leafroll virus(PLRV), PVY, potato virus A (PVA), PVX, potato virus M (PVM), and potato virus S (PVS) being the most significant in terms of distribution and their impact on yield across the globe. In recent times, several viruses that have re-emerged or newly emerged have posed a significant threat to potato crops, and if left unaddressed, have the potential to considerably curtail production in the future. Therefore, it is crucial to take immediate action to mitigate their impact and prevent their spread.

The initial discovery of potato leafroll virus in Germany in 1906 by Appel[15] did not identify its cause, but Quanjer et al. later established it as an infectious agent in 1916[16]. Schultz and Folsom then identified aphids as the vector for the disease in 1923[17]. In the Netherlands, Van Slogteren pioneered the use of serological methods for potato virus detection[18], producing antisera for Potato virus X (PVX) and Potato virus S (PVS)[19]. Since then, significant advances have been made in virus detection and the production of transgenic plants resistant to viruses[20]. While many Mediterranean countries import certified seed potatoes, their own seed production is mainly used for planting in the autumn and winter, while imported seeds are used for early and late spring plantings. Certified seed production is carried out in some Mediterranean countries, but the process of certification over 6-7 generations as practiced in Northern European countries is not applicable in Mediterranean countries due to the climate and growing conditions. In warmer climates, a dormancy period of 3-4 months for the potato tuber does not allow the operation of the European certification scheme. Despite this, viruses such as PVY and Potato leafroll virus (PLRV) can still be constituting in the second crop after planting certified seed, particularly if infected plants are left in the field or in neighboring plots. The seed potato industry in the Basque Country and surrounding areas has been significantly disrupted by high rates of PVY infection leading to an average increase in losses from 1% to 10% in a single round of field propagation[21].

1.4 Detection tools for plant pathogen detection

Potato is a highly susceptible host of various plant viruses, leading to significant yield losses as stated in the above section. Unfortunately, there is no direct chemical treatment available globally to control these viruses. Therefore, the first line of defense against them is to inhibit their establishment, evolution, and dispersal. Effective detection techniques are crucial to prevent their spread, and to produce healthy seed potatoes, strong virus indexing is the only option. With the globalization of trade by free trade agreement, the detection of potato viruses has become a concern for national quarantine

services to ensure the safe movement of potato germplasm across borders.

Detection and identification of potato viruses is not an easy task, as they are host-specific and require appropriate detection methods from sampling to the final step. Molecular diagnostic techniques may be the preferred choice for disease-free seed potato production. However, for epidemiological and aetiological studies, biological indexing, serological, and electron microscopy techniques are essential.

At the biological level, addressing plant pathogen diseases involves the rapid and precise identification of the causative organism, accurate estimations of symptom severity and yield impact, and identification of virulence mechanisms. Disease prevention can then be achieved through the reduction of the pathogen inoculum, inhibition of virulence mechanisms, and the promotion of genetic diversity in crops. In many cases, plants may be infected with more than one pathogen, which results in more complicated and severe diseases. Advanced disease identification and prevention techniques are critical for minimizing damage and maximizing yield to ensure agricultural sustainability. Currently, a variety of detection methods are available, ranging from laboratory-based techniques to point-of-care detection of plant pathogens in the field. Polymerase chain reaction (PCR), fluorescence-linked immunosorbent assay (FLISA), enzyme-linked immunosorbent assay (ELISA), flow cytometry, and gas chromatography-mass spectrometry (GC-MS) are among the laboratory techniques utilized for the direct detection of plant diseases. However, there are challenges in terms of mainstream adoption of these techniques, including the need for significant time for sample preparation and analysis, expensive equipment, frequent cross-contamination, and the requirement for specialized personnel to handle sophisticated instruments.

Various methods have been developed and evaluated to detect plant pathogens, including nucleic acid-based detection, protein-based detection, multiplex detection, and nanotechnology-based sensors for plant antigen screening. Recent advances in nanosensor technology have allowed the downsizing of biosensors for detecting pathogen presence in plants, utilizing antibodies, DNA, and other bio-responsive complexes. The use of nano-biosensor technology has revolutionized plant diagnostic systems by enabling non-destructive, minimally invasive, cost-effective, and user-friendly devices that provide enhanced detection limit, sensitivity, specificity, and on-site detection of plant diseases. The typical blueprint that is presently used for plant disease detection is illustrated in **Figure 1.2**

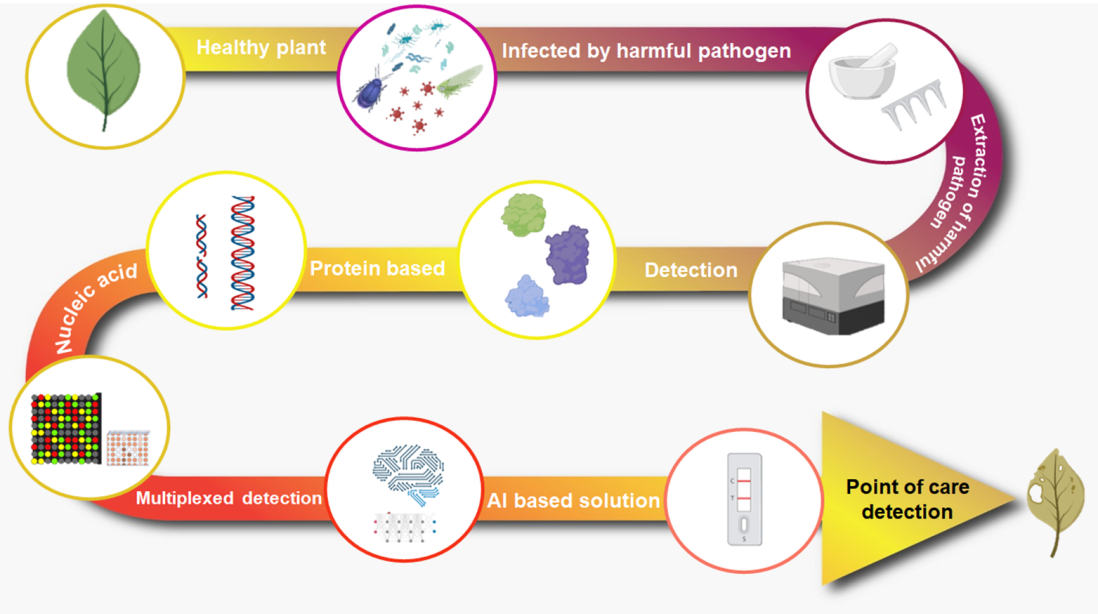


Figure 1.2: A blueprint for plant pathogen detection over the years[5]

1.5 Detection of plant diseases using portable sensors

1.5.1 Microfluidic-based portable sensor for plant pathogen detection

Point-of-care testing (POC) has been around for a long, but it has only lately gained popularity in the field of phytodiagnostics. Farmers can make quick judgments in emergency scenarios because to point-of-care diagnostics. Because the findings are accessible in minutes, point-of-care testing may now screen bigger samples. The **Figure1.3** depicts various biological strategies used to design POC devices for plant pathogen detection.

Microfluidic sensors are increasingly being recognized as a critical tool for a variety of analytical applications, particularly for the detection of pathogens in food, water, and environmental samples. These devices offer several advantages over conventional detection methods, including high sensitivity, rapid detection, and low sample volume requirements. Additionally, microfluidic sensors can be easily integrated with a variety of sensing modalities, including optical, electrochemical, and acoustic sensors. Perhaps most importantly, microfluidic sensors are now being designed to be portable, which greatly enhances their utility in field settings. The ability to perform rapid, sensitive, and specific detection of pathogens in the field is essential for the prevention and control of infectious diseases in both human and plant populations, making microfluidic

sensors a crucial technology for advancing public health and food security. There are several examples of microfluidic sensors, including:

1. Lab-on-a-chip (LOC) sensors - These are microfluidic devices that integrate a variety of microfluidic components with sensing technologies to create a complete analytical system. They are being used for a wide range of applications, including environmental monitoring, medical diagnostics, and food safety testing.

2. Paper-based microfluidic sensors - These are inexpensive and portable sensors that use paper as the substrate for the microfluidic channels. They are being developed for a variety of applications, including pathogen detection, environmental monitoring, and medical diagnostics.

3. Droplet-based microfluidic sensors - These are sensors that use small droplets of fluid to perform complex chemical reactions and analyses. They are being developed for a variety of applications, including high-throughput screening, drug discovery, and medical diagnostics.

4. Microcantilever sensors - These are sensors that use microcantilevers as the sensing element. They can detect a variety of analytes, including biomolecules, gases, and liquids. They are being developed for a variety of applications, including environmental monitoring, medical diagnostics, and food safety testing.

Overall, microfluidic sensors are becoming increasingly important in many areas of research and development due to their ability to perform complex biochemical assays with high sensitivity, accuracy, and speed, as well as their potential for portability and affordability. The **Figure1.3** depicts various biological strategies used to design POC devices for plant pathogen detection.

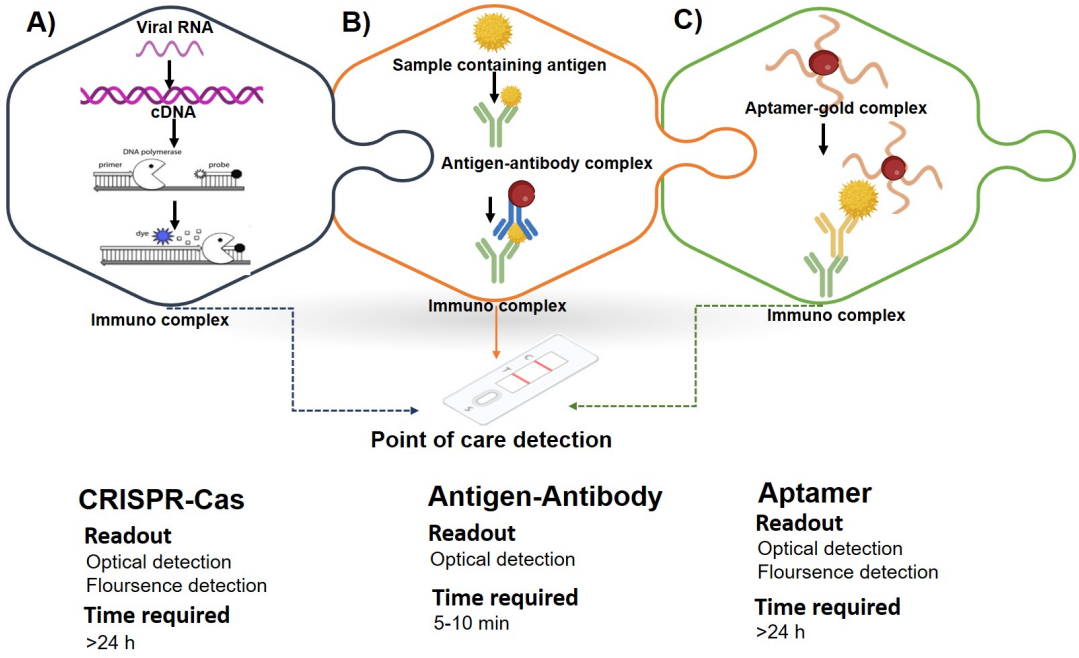


Figure 1.3: Point-of-care integration a) CRISPR-Cas based testing, b) Antigen-antibody based testing c) Aptamer based detection for plant pathogen detection [5]

1.5.2 Multiplexed point-of-care diagnostic platform for plant pathogens

Multiplex detection methods are becoming increasingly popular in the field of plant pathogen screening. With the ability to detect and quantify multiple analytes at the same time, multiplex assays offer many advantages over traditional single-analyte tests, including increased efficiency, accuracy, and cost-effectiveness. In plant pathogen screening, the ability to detect multiple pathogens in a single test is essential, as plants can be susceptible to various diseases simultaneously. Multiplex assays have been developed to detect a wide range of plant pathogens, including bacteria, fungi, and viruses. These assays rely on different techniques, such as microsphere immunoassays, real-time PCR, and RPA, among others.The use of multiplex detection methods has been widely adopted in the seed and plant industries, where early and accurate detection of plant pathogens is critical to prevent the spread of diseases and minimize crop losses. These methods not only facilitate the screening of multiple pathogens simultaneously but also offer the potential for high-throughput screening, enabling the testing of large numbers of samples in a short amount of time. In addition to their applications in pathogen screening, multiplex assays have also been utilized in other areas, such as food safety and clinical diagnostics. They have shown great promise in the detection of multiple analytes, including bacterial and viral infections, genetic disorders, and cancer biomark-

ers. Overall, multiplex detection methods offer many benefits in the detection of plant pathogens, including increased efficiency, accuracy, and cost-effectiveness. As technology continues to advance, these methods are expected to become even more accessible and widely used in the field of plant pathology and beyond.

1.6 Research gap

From an agricultural perspective, the development of reliable, selective, and sensitive plant virus biosensors is of paramount importance to safeguard the health of crops and ensure food security. Despite their promising performance in lab conditions, the current generation of plant virus biosensors still lack the necessary robustness and selectivity for real-world applications. The inherent complexities associated with multicomponent mixtures have further compounded the challenges associated with the identification of specific plant viruses. Despite ongoing research endeavors, which are extensive when talking about basic research and identification of potential biomarkers associated with the plant virus, little to no efforts are in the pipeline considering only a limited number of plant virus biosensors that have been successfully deployed in practical settings. Nonetheless, these biosensors can still provide valuable information as screening devices for farmers, as they are capable of detecting the trace level of viral titer. Moreover, plant virus biosensors are the most cost-effective, rapid, and sensitive approach for determining the toxicity of agricultural samples, with no suitable alternatives available at present. Moving forward, it is envisioned that these biosensors will play an increasingly prominent role in the agricultural sector by complementing or even replacing complex and expensive chromatographic methods which are currently extensively utilised. In particular, the primary application of plant virus biosensors will be to screen for multiple analytes in the longer term, thereby facilitating timely and informed decisions regarding crop management and food safety[22].

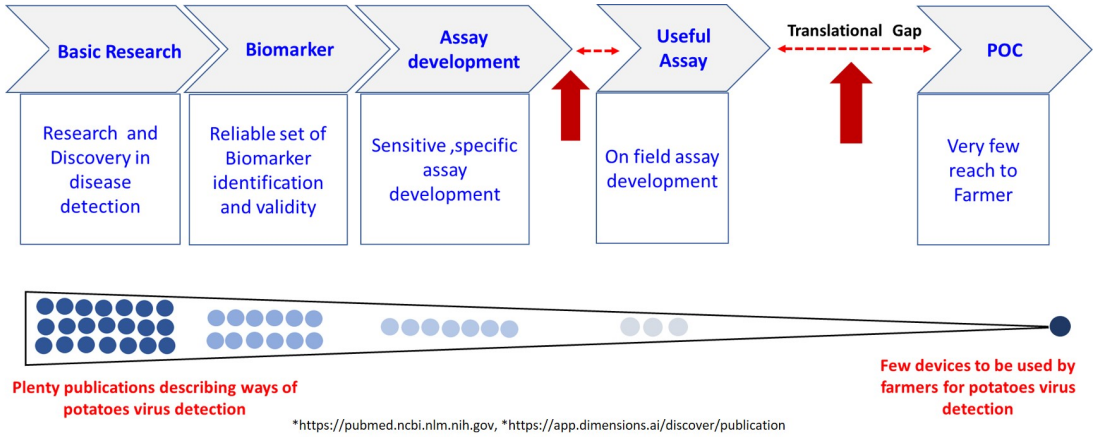

Basic Research
Biomarker
Assay development
Useful Assay
Translational Gap
POC
Research and Discovery in disease detection
Reliable set of Biomarker identification and validity
Sensitive ,specific assay development
On field assay development
Very few reach to Farmer
Plenty publications describing ways of potatoes virus detection
Few devices to be used by farmers for potatoes virus detection
*https://pubmed.ncbi.nlm.nih.gov, *https://app.dimensions.ai/discover/publication

Figure 1.4: Identified research gap

1.7 Scope: Unmet Need

There is an unmet need for ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable) diagnostics in agriculture. Unlike human and animal health, where diagnostic tools have been developed and are widely used, there has been a lack of investment in developing affordable and user-friendly diagnostic tools for plant diseases. This is despite the fact that plant diseases can cause significant losses to farmers and threaten global food security.

Current diagnostic methods for plant diseases often require expensive laboratory equipment and skilled personnel, making them inaccessible and unaffordable for small-scale farmers, who make up the majority of agricultural producers in developing countries. Furthermore, these methods can be time-consuming and may not provide timely results, leading to delays in implementing control measures.

There is a growing recognition of the need to develop ASSURED diagnostic tools for agriculture. This would require a collaborative effort between scientists, industry, and policymakers to identify the most pressing diagnostic needs, develop innovative and affordable technologies, and ensure that these tools are accessible and usable by farmers.

ASSURED diagnostics in agriculture would have significant benefits, including early detection and control of plant diseases, reduced use of pesticides and other chemical inputs, improved food safety, and increased productivity and profitability for farmers. As such, there is a pressing need to invest in research and development to bring these diagnostic tools to fruition and ensure that they are widely available to those who need them the most.

Objective

From the rigorous research and extensive review of literature, we have set the following objective to fulfill the goal to develop a cost-effective nano diagnostic platform for potato virus detection.

1. Synthesis of nanoparticle by using safe by design approach for developing assay
2. Development of paper microfluidic and electrochemical-based testing kit for detection of Potato virus
3. Optimization and on-field validation of the developed kit or assay
4. Simultaneous detection of multiple viruses.

Chapter 2

Development of Vertical flow immunoassay for detection of PVY

2.1 Introduction

Potato is a non-grain food crop widely grown and consumed globally. It is also prone to several viral diseases that cause severe damage to crop yield, with Potato virus Y (PVY) being a particularly concerning pathogen. It infects potato cultivars in various territories and is economically significant, requiring the development of a rapid and sensitive on-site testing method to detect the virus[23] [24]. Given the global population growth and rising demand for food, addressing plant viruses and improving crop productivity is a pressing issue that requires immediate attention and action. Several serological and molecular techniques have been put forth for detecting PVY, such as immunosorbent electron microscopy, ELISA[25], reverse-transcription polymerase chain reaction amplification (RT-PCR)[26,27], real-time-PCR[28,29], and reverse-transcription loop-mediated isothermal amplification (RT-LAMP)[30,31]. Despite the benefits of these methods, including their accuracy, they are not practical for on-site detection due to their time-consuming nature, requirement for trained personnel, and high-end equipment. The limitation of these methods has constrained their application in the point-of-care setting[32,33,34]. However, paper-based microfluidic platforms, which are low-cost and simple to use, have become increasingly prevalent for detecting and diagnosing plant diseases[35,36]. Lateral flow assays are particularly promising as an easy-to-use platform that can surmount the drawbacks of molecular-based detection techniques[37,38]. These assays have demonstrated an ability to overcome problems such as complexity, and recent advances have focused on developing novel signal-amplification strategies, applying new labeling techniques, enhancing quantification systems, and enabling simultaneous detection to improve their effectiveness. However, developing lateral flow

assays has been arduous due to the difficult fabrication process and the necessity of using multiple membranes[39,40]. Furthermore, traditional lateral flow assays are limited by the hook effect, which occurs when excessive antigens interfere with the assay. This effect prevents the binding of the gold antibody nanoconjugate and blocks the formation of the immunocomplex[41,42]. Previously, our group had conducted research on using gold nanoparticles (GNP) to create nanosensors for detecting metals, biomolecules, and diseases[43,44]. To overcome these limitations, we have designed and developed a field deployable vertical flow immunodevice (VFID) for the detection of PVY virus. Several key factors for optimizing the immunodevice, such as nanoconjugate preparation, blocking buffer, and nanoconjugate volume, were determined to develop a highly specific and reliable VFID for the detection of PVY virus. Additionally, we studied biosensor construction parameters, including antibody concentration and resuspension buffer. We tested the VFID accuracy by analyzing potato leaf samples obtained from a potato farm. Our findings suggest that the developed VFID will be a valuable tool for the rapid on-site detection of PVY virus. In summary, our work has addressed the limitations of traditional lateral flow assays and developed an innovative VFID device that is highly specific and reliable for the detection of PVY virus.

2.2 Assay Mechanism and Interpretation of VFID

The vertical flow immunodevice (VFID) for the detection of Potato Virus Y (PVY) antigen consists of a nitrocellulose membrane (NCM) and absorbent pads placed in a 3D printed plastic housing. Assembly of the VFID is achieved by carefully placing the NCM at the center of the device and then layering adsorbent pads below it. One of the key advantages of the VFID is its reduced assay time compared to traditional lateral flow assays, which is attributed to the faster capillary action enabled by its vertical flow mechanism.

In terms of its working mechanism, the nitrocellulose membrane of the immunodevice is immobilized with capture PVY antibody, which specifically binds to the PVY antigen. When a sample containing the PVY antigen is introduced to the device, it binds to the capture antibody that is immobilized on the NCM. The detection of the bound PVY antigen is subsequently achieved through the binding of gold nanoparticles (GNP) conjugated with Anti-PVY antibody, also known as the detection antibody. This step completes the immunocomplex by forming a sandwich consisting of a PVY capture antibody, PVY antigen, and GNP-Anti-PVY, and results in a visual appearance of a red-colored dot, which indicates the presence of the PVY antigen. In the specific case of the VFID for PVY antigen detection, the assay format is modified to a vertical flow mechanism, which offers improved performance and sensitivity over traditional lateral

flow assays. The actual test results of the VFID for PVY antigen detection are depicted in **Figure 2.1**.

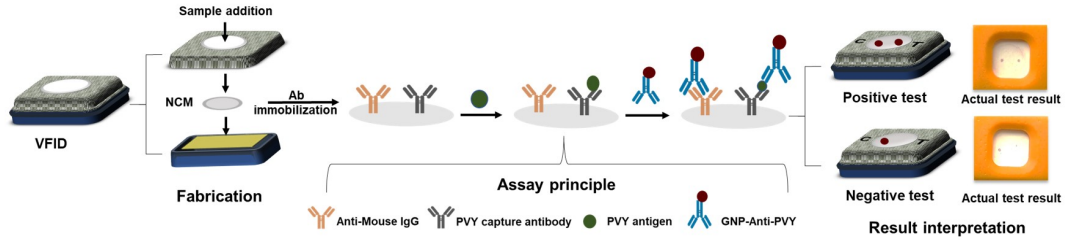
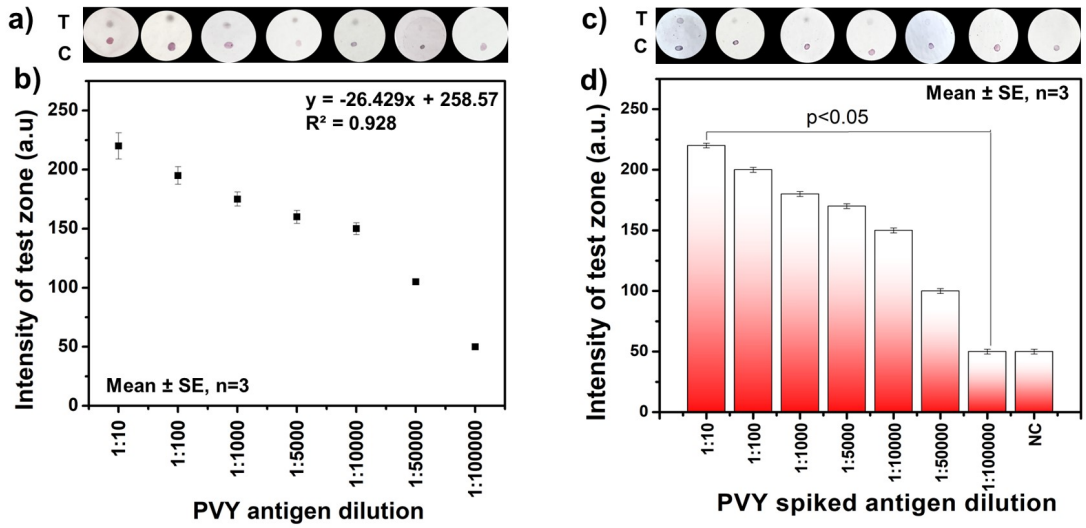


Figure 2.1: Assay mechanism and test result interpretation of VFID[45]

2.3 Results and Discussion

To evaluate the ability of VFID to detect PVY, dilutions of PVY antigen were applied to the test strips under optimized experimental conditions. Dilutions ranging from 1:10 to 1:100,000 were tested, and the results are shown in **Figure 2.2a**. The lowest detectable intensity of PVY virus was observed at the dilution of 1:10,000. A calibration curve was then plotted by correlating the intensity of the test spot with the dilution of PVY ranging from 1:10 to 1:100,000. The regression equation was determined to be y = 26.429x + 258.57 with a coefficient of regression of R^2 = 0.928 **2.2b)**. To further assess the detectability of VFID for PVY, positive control of PVY antigen was spiked into healthy potato leaves extract at dilutions ranging from 1:10 to 1:100,000 under optimized experimental conditions **(Figure 2.2c)**. The results confirmed the ability of VFID to detect PVY at a dilution of 1:10,000 with high specificity and sensitivity. Overall, these findings demonstrate the effectiveness of VFID as a reliable diagnostic tool for the detection of PVY in potato leaf samples. The effectiveness of the VFID assay for detecting PVY was validated via spiking of healthy potato leaf extract with diverse dilutions of the positive control of PVY antigen, spanning from 1:10 to 1:100,000. The intensity of the test spot was ascertained for each dilution, revealing that the VFID exhibited the ability to detect PVY up to a dilution of 1:10,000, which is comparable to the standard antigen**(Figure 2.2d)**. The intensity of the test spot decreased linearly with decreasing antigen dilution, signifying the absence of a hook effect. The assay was replicated thrice, and the relative error was calculated to determine the accuracy of VFID.



e)

PVY antigen dilution	1:10	1:100	1:1000	1:5000	1:10000	1:50000	1:100000
% Relative error	2.5	0.56	1.25	0.54	2.08	0.27	1.05

Figure 2.2: Detectability of VFID with different dilutions of standard PVY antigen and spiked PVY (mean ± S.E). a) Typical images of VFID depicting different dilutions of standard PVY antigen dilution; b) Graph of the intensities of VFID test spots versus PVY antigen dilution (n = 3); c) Typical images of VFID after spiking with different dilution of virus with potato leaves extract; d) Graph of the intensities of the VFID test spots versus spiked dilution of the potato leaves extract (n = 3); e) % Relative error for each spiking dilution. (F-test (F (6,14) = 1409.17, $p < 0.05$))[45]

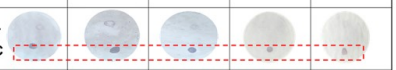
To ensure the robustness of the developed kit, it was validated using potato leaf samples collected from two major potato-producing regions in Gujarat, India, and analyzed over a period of 50 days, at 10-day intervals. A total of 10 samples were collected (n=10) overall, with 5 samples obtained from each region, and subjected to analysis and validation using our developed VFID. It is noteworthy that two out of five samples from region 1 tested positive for PVY antigen on Day 40 and Day 50, which emphasizes the effectiveness of our method in detecting the virus in potato samples. After conducting the tests, it was found that three of the collected samples from both regions tested negative for PVY antigen. As a result, no test spots appeared, as illustrated in **Figure 2.3a and 2.3b**. However, for two samples from region 2 on Day 10 and Day 20, faint spots appeared, but their intensity was found to be similar to that obtained for the negative control using ImageJ software. It is important to note that the samples that resulted in positive signals had varying intensities above the positive control signal, indicating different amounts of PVY antigen load in the samples from both regions, as demonstrated in **Figure 2.3c and d**. To evaluate the performance of VFID, various statistical parameters such as standard deviation, coefficient of variance, and relative error were calculated for each test conducted in both regions. In region 1, the average standard

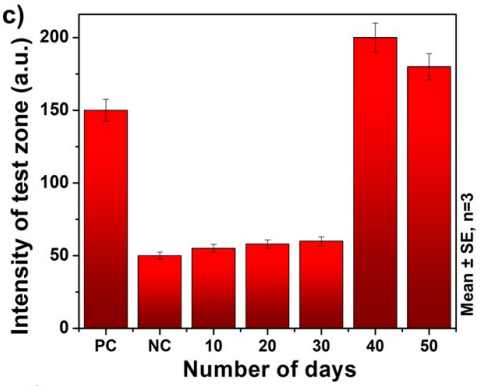
deviation was determined to be 0.99, with a coefficient of variance of 0.66 and a relative error of 0.57%. Similarly, in region 2, the average standard deviation was 1, with an average coefficient of variance of 0.55 and a relative error of 0.63%. These statistical parameters help to establish the reliability and accuracy of the VFID in detecting PVY virus in potato leaf samples.

a)

Region 1	Number of days				
	10	20	30	40	50
Potato leaf samples					
VFID					
ELISA	Negative	Negative	Negative	Positive	Positive
VFID (Statistical analysis)					
Standard deviation	1.65	0.81	1.24	0.81	0.47
Coefficient of variance	1.42	0.74	0.60	0.37	0.20
% Relative error	0.94	0.47	0.72	0.47	0.27

b)

Region 2	Number of days				
	10	20	30	40	50
Potato leaf samples					
VFID					
ELISA	Negative	Negative	Negative	Negative	Negative
VFID (Statistical analysis)					
Standard deviation	0.47	0.57	1	0.57	2.8
Coefficient of variance	0.42	0.48	0.50	0.23	1.16
% Relative error	0.27	0.33	0.57	0.34	1.67



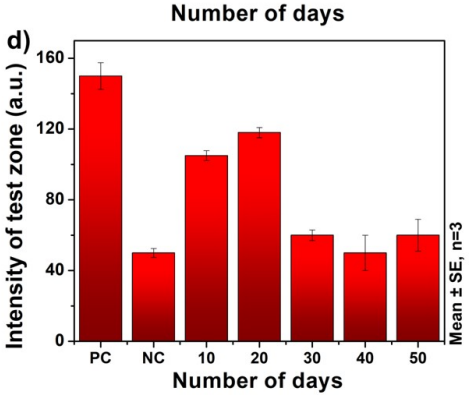


Figure 2.3: Visual detection of PVY with VFID (mean ± S. E, n = 3) from a) region 1 and b) region 2; c,d) Graph of the intensities of the VFID test zones for each tested sample from region 1 and region 2 at $p < 0.05$ (NC, negative control (resuspension buffer solution); PC, positive control (1:10 000 dilution of PVY antigen))

To further validate of the developed VFID for on-site detection, ELISA tests were performed in triplicate and the relative error was determined. The t-test was used to statistically verify the positive and negative results of the VFID in comparison to the positive and negative controls, respectively. The t-test showed no significant differences between the groups ($p < 0.05$). The good correlation between ELISA and VFID confirmed the suitability of the developed VFID for on-site detection.

2.4 Conclusion

In conclusion, we have designed and developed a highly effective and rapid vertical flow immunodevice (VFID) for the detection of PVY virus. We carefully optimized several key parameters such as the nanoconjugate, blocking buffer, antibody concentration, resuspension buffer, and nanoconjugate volume to ensure accurate and reliable on-field screening of PVY. Importantly, the VFID enables visual detection of PVY within 3-4 minutes, without requiring any specialized instruments, making it a convenient and accessible diagnostic tool. The excellent accuracy and performance of the VFID are evidenced by a low relative error ranging between 0.2 and 2%. We also tested the reliability of the VFID using potato leaf samples from various farm regions, demonstrating its ability to accurately detect PVY in real-world settings. Therefore, our developed immuno device presents a valuable diagnostic tool for the detection of potato viral disease, providing farmers and agricultural professionals with an affordable, user-friendly, and effective solution for on-field screening.

Chapter 3

Development of Electrochemical immunosensor for detection of PAMV

Introduction

Potatoes are susceptible to around 40 viruses, with the majority being single-stranded positive-sense RNA viruses. However, only a handful of viruses, including potato leaf roll virus (PLRV), potato aucuba mosaic virus (PAMV), potato virus Y (PVY), potato virus A (PVA), and potato virus X (PVX), are found in potato growing areas worldwide[46,47,48]. Current potato viral disease protection relies on a limited number of diagnosis technologies, such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assays (ELISA), fluorescence in situ hybridization (FISH), immunofluorescence (IF), and flow cytometry (FCM)[49,50,51]. However, most of these detection technologies are laboratory-based and require skilled technicians to operate the instruments. Furthermore, centralized diagnostic laboratories are often located far from agricultural areas. To monitor plant health, cost-effective, field-deployable, and highly sensitive disease detection systems are crucial.

The screen-printed carbon electrode (SPCE) is a popular choice for electrochemical immunosensing due to its ease of use and low cost. Nanomatrix supports like gold nanoparticles, graphene oxide, and magnetic nanoparticles have enhanced immunosensor performance[52,53,54,55]. Proper orientation of active antibodies is critical for sensitivity and specificity, reducing nonspecific binding and improving electron transfer rates[56,57,58]. Metal-based nanoparticles have been utilized to enhance the sensitivity of immunosensors. However, the effect of size and shape on antibody immobilization strategy has been overlooked[59,60]. To address this, we developed a shape-dependent nanomatrix supported antibody immobilization strategy for detecting potato aucuba mosaic virus (PAMV) using a covalent binding approach. We compared the performance

of EDC-NHS activated spherical gold nanoparticles and reduced graphene oxide (rGO) nanosheets. We also cross-validated the immunosensor performance using other plant biomarkers such as PVY and PVA, while evaluating surface coverage and percent active antibody. To our knowledge, this is the first report of such work for detecting PAMV.

3.1 Immobilization strategy

The electrochemical immunosensor proposed here utilized an effective antibody immobilization strategy that aimed to enhance specific binding while suppressing non-specific binding. The impact of the antibody immobilization strategy on the nanomatrix support with a shape-dependent factor was studied using SPCE to evaluate factors such as antibody binding activity, surface area coverage, limit of detection, and accuracy. The antibody was immobilized on SPCE by passive adsorption and covalent attachment on rGO nanosheets and spherical AuNP. Carboxylate groups were activated using EDC-NHS chemistry. Passive adsorption was driven mainly by ionic bonds, electrostatic and hydrophobic interactions, and van der Waals forces, resulting in random orientation of the antibody.

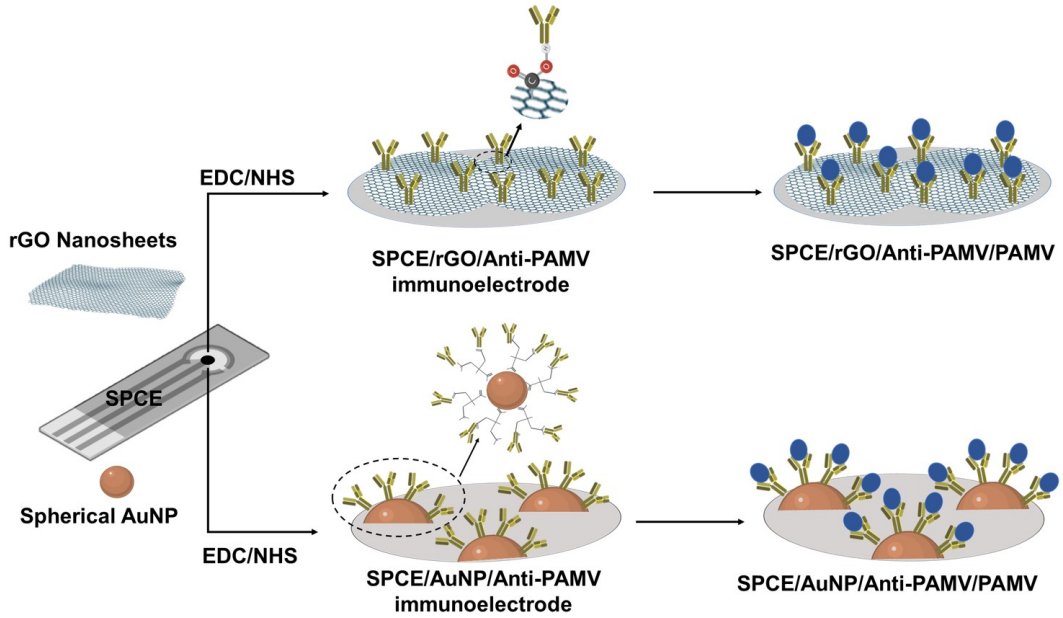


Figure 3.1: Schematic representation: antibody immobilization strategy via. gold nanoparticle (AuNP) and reduced graphene oxide (rGO), followed by fabrication of screen printed electrode (SPCE), SPCE/AuNP and SPCE/rGO based immunoelectrode for PAMV detection

Covalent immobilization provided high sensitivity through a definite orientation for the antibody on the modified electrode surface. The amine (-NH_2) groups present on the

lysine amino acid side-chain on the antibody surface were used for covalent binding to achieve oriented immobilization. In accordance with the immobilization strategy, EDC formed neutral amide bonds between carboxyl groups of SPCE/AuNP and SPCE/rGO with primary amines of anti-PAMV antibody, significantly improving sensitivity and specificity **Figure 3.1**. Spectroscopic and electrochemical techniques confirmed the effect of the immobilization strategy.

3.1.1 Quantitative capability of PAMV immunosensor based on active antibody

3.1.1.1 Electrode surface area and antibody coverage

The surface area of the immunosensor was determined before and after surface modifications on the working electrode. The electrochemically active surface area of the unmodified SPCE was calculated to be 3.8 x 10^{-5} cm^2 using the slope of cyclic voltammogram peak currents versus the square root of the scan rate ($v^{1/2}$). After modification with AuNP and rGO, the SPCE surface areas were found to be 4.52 x 10^{-5} cm^2 and 5.32 x 10^{-5} cm^2, respectively. The surface areas before and after modifications were also calculated based on the theoretical dimensions of antibody coverage (14.5 × 8.5 × 4 nm^3)[61]. The antibody coverage on the unmodified SPCE was determined to be 0.7± 0.07 x 10^5, while the AuNP and rGO modified SPCE had coverages of 6.09 ± 0.07 x 10^5 and 5.59 ±0.06 x 10^5, respectively. These findings indicate that AuNP and rGO modified SPCE offers a larger active surface area for antibody binding.

3.2 Percentage availability of active antibody

To assess the active antibody availability and achieve high sensitivity, the performance of the PAMV immunosensor was evaluated by calculating the percentage of active antibodies immobilized on the surface[62]. The active antibody immobilization is crucial as it allows the Fab region of the antibody to be accessible for binding with the antigen, resulting in increased sensitivity. The percentage availability of active antibodies was determined using DPV by comparing the real current response with the ideal current response, which represents the accessibility of the Fab region of the immobilized antibodies for binding with the antigen. The PAMV antigen concentration of 10^{-1} M was used as a standard for SPCE, SPCE/AuNP, and SPCE/rGO. The SPCE/AuNP and SPCE/rGO showed an increase in current due to enhanced electron transfer rate, whereas the unmodified SPCE showed a relatively lesser current response. The real current was calculated by subtracting the current response without antigen from the cur-

rent response after adding the highest antigen concentration. The percentage availability of active antibodies was then calculated as

$$\%Availability = Realcurrent/Idealcurrent * 100 \quad (3.1)$$

The formula to calculate the real current is defined as the difference between the current response after the addition of the highest antigen concentration and the current response without antigen. On the other hand, the ideal current response is the expected value when the antibodies are fully accessible for binding with two antigen molecules.

% Ideal current = Current without antigen x 2/ (Wt. of Antibody in kDa /Wt. of Antigen in kDa) (3.2)

For unmodified SPCE, the real current was determined to be 4.79 μA while the ideal current response was calculated to be 63.59 μA. In contrast, for SPCE/AuNP, the real and ideal current responses were found to be 49.31 μA and 140.44 μA, respectively. For SPCE/rGO, the real and ideal responses were 67.77 μA and 81.04 μA, respectively. Therefore, the percentage availability of active antibodies was calculated to be 35% for SPCE/AuNP, 26% for SPCE/rGO, and 7% for unmodified SPCE. The results showed that SPCE/AuNP had the highest percentage of active antibody availability, making it a suitable choice for the detection of PAMV.

3.3 Sensitivity of fabricated immunosensor

The sensitivity of the newly developed immunosensor was evaluated at various PAMV analyte concentrations using differential pulse voltammetry (DPV) ranging from 0.8 M (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-6}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, and 10^{-13}) for SPCE/AuNP/PAMV and SPCE/rGO/PAMV, respectively, under optimal conditions. The DPV scans of all immunoelectrodes showed a decrease in current response upon antigen binding, as illustrated in **Figure 3.2 a, b**. This decline was due to hindered electron transfer resulting from the formation of an insulating layer caused by increased antigen binding. The immunosensor showed a linear response in the concentration range of the analyte. The response signals of the SPCE/AuNP/Anti-PAMV and SPCE/rGO/Anti-

PAMV immunoelectrodes were fit into the following linear regression equations:

$$Ip = -0.0494(PAMV) + 20.73, R^2 = 0.9478 \tag{3.3}$$

$$Ip = -0.03577(PAMV) + 22.40, R^2 = 0.98048 \tag{3.4}$$

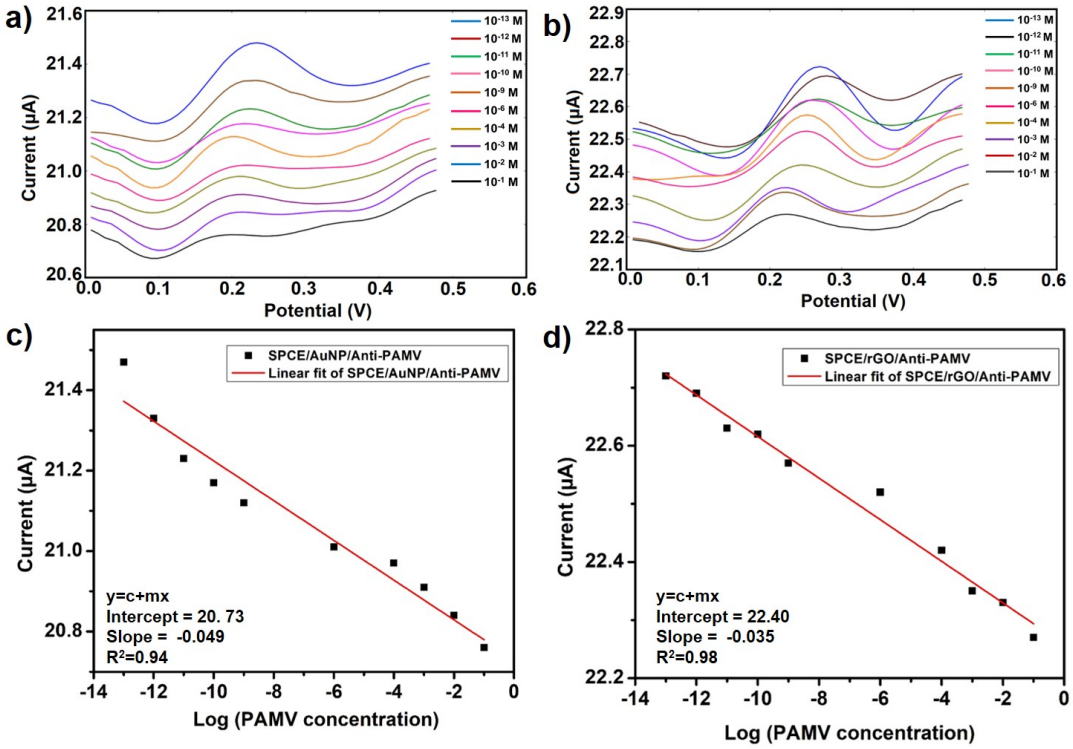


Figure 3.2: DPV response of immunoelectrode in different standard concentration of PAMV antigen (10^{-1} to 10^{-13} M) on a) SPCE/AuNP/Anti-PAMV, b) SPCE/rGO/Anti-PAMV immunoelectrode; calibration curve of PAMV concentration in the linear range (10^{-1} to 10^{-13} M) on, c) SPCE/AuNP/Anti-PAMV and d) SPCE/rGO/Anti-PAMV immunoelectrode

The limit of detection (LOD) of the SPCE/AuNP/Anti-PAMV and SPCE/rGO/Anti-PAMV immunoelectrodes was determined as mentioned by (Tiwari et al. 2016). The LOD was calculated using the formula[63]

$$LOD = k.\sigma/S \tag{3.5}$$

where k is the confidence level parameter (k = 3 with a statistical confidence of 99.6%), is the standard deviation of blank measurements (taken as RSD for 5 measurements), and S is the sensitivity of the electrode which is equal to the slope (/) of the current vs dilution plot. The LOD of the SPCE/AuNP/Anti-PAMV immunoelectrode

was determined to be at a concentration of 0.96 x 10-12 M, whereas the LOD of the SPCE/rGO/Anti-PAMV immunoelectrode was determined to be at a concentration of 0.065 x 10^{-12} M.

3.4 Conclusion

A rapid, highly selective and sensitive electrochemical immunosensing platform has been successfully developed for the early detection of PAMV. The shape-dependent immobilization of antibodies was identified as a key factor for the platform efficacy, with a high degree of surface coverage and stability being crucial for effective immobilization of active antibodies. The SPCE/AuNP/Anti-PAMV immunoelectrode exhibited the highest antibody percent availability of 35%. The LOD was found to be 0.96 x 10^{-12} M for SPCE/AuNP/Anti-PAMV and 0.065 x 10^{-12} M for SPCE/rGO/Anti-PAMV. The immunosensor demonstrated excellent performance in spiked potato leaf samples, with an average recovery of 86.4% and 96.9 % for SPCE/AuNP and SPCE/rGO, respectively. The stable covalent conjugation and high antibody percent availability make SPCE/AuNP a promising electrochemical immunosensor. This innovative immunosensor can be utilized as a potential and portable nano diagnostic platform for on-site plant health monitoring.

Chapter 4

Development of electro-chemical immuno-sensor for diverse bio-markers

4.1 Introduction

The current POC platform employs paper-based assay kits, smartphone-enabled operations, and lab-on-a-chip devices, as they offer superior sensitivity, information content, and suitability for disease diagnosis. These technological advancements have the potential to revolutionize the field of diagnostic testing, making it more accessible and efficient for patients and healthcare providers alike. End users primarily rely on paper-based platforms for disease diagnostic purposes. In recent years, there has been significant progress in the development of paper-based lateral flow point-of-care (POC) tests that can detect specific biomarkers like human chorionic gonadotropin (hCG) and human immunodeficiency virus (HIV). the detection strategy used in these tests can only identify one analyte, which limits their potential for detecting multiple biomarkers. Although there are several POC signal readout strategies available, including colorimetry[64], fluorimetry[65], electrochemiluminescence[66], and magnetic immunoassays[67], electrochemical-based signal readout is an attractive option for immunosensor platforms due to its higher quantitative accuracy and lower susceptibility to false results caused by color interference. Despite this, the use of electrochemical sensing for the simultaneous detection of diverse biomarkers has not been widely explored[68,69]].To detect different biomarkers, the most suitable method is to develop paper-interfaced electrochemical immunosensors. These sensors have paper interfaces that offer stability and core functionality, which greatly improve sensor performance and attract the target analytes with little background or nonspecific responses. Nevertheless, only a few paper-

based POC devices have been tested for detecting multiple biomarkers using universal detection strategies. Therefore, there is still much to discover in terms of developing paper-interfaced electrochemical sensors that can detect diverse biomarkers. The use of a streptavidin-biotin-based approach is highly appealing when it comes to electrochemical detection of various biomarkers due to its adaptable recognition strategy[70,71]. For the past 25 years, interactions between streptavidin and biotin have played a significant role in the evolution of immunoassays[72]. The advantages that come with these interactions include their high stability and selectivity[73]. Additionally, proteins can be firmly bound together with the help of biotin, which can be easily covalently coupled with them. This feature also facilitates the formation of immunocomplexes, which is essential for the development of immunosensors capable of detecting a wide range of biomarkers[74,75,76].

Furthermore, to enhance the sensitivity of the immunensor, the electrode surface can be modified with conductive nanomaterials to increase the electron transfer rate[77]. One such nanomaterial that is highly attractive for the electrochemical sensing of biomolecules is Prussian blue nanocubes (PBNCs). PBNCs compounds have been a topic of interest in the last decade owing to their nature and applications. They are highly advantageous as they possess flexible configurations, tunable physical, and chemical attributes, which can be governed by controlling size, surface and morphology, porosity, reduced density, and the ability to act as multifunctional agent. Significant changes in the physicochemical properties and improved electrochemical performance have led the research community to explore the nanostructures derived from PBNCs. Also their biocompatibility, high electrocatalytic activity, and low overpotential detection[78,79,80], make these nanomaterials suitable for use in sensor fabrication. Furthermore, Prussian blue nanocubes (PBNCs) can be biofunctionalized with avidin through electrostatic self-assembly, and they maintain their biomaterial properties even after functionalization, making them an excellent option for sensing applications. Hence, we developed a novel immunodevice utilizing Prussian blue nanocubes and a paper disc (PD) for simultaneous electrochemical detection of diverse biomarkers. The immunodevice was fabricated with a streptavidin-biotin detection mechanism to target varied biomolecules with the aid of a replaceable PD. The PD serves a crucial role in the detector, enabling its use for detecting diverse biomolecules while maintaining its universality. The signal amplification strategies used in the immunodevice impart high sensitivity and low false-negative outcomes. We assessed the detection ability of the immunodevice using Potato virus X (PVX) as a model biomarker, and electrochemical techniques were used for analysis. The immunodevice exhibited excellent specificity in detecting PVX, discerning it from similar biomarkers such as Potato aucuba mosaic virus (PAMV), Potato virus Y (PVY), and Potato virus A (PVA). The versatility of the PD-interfaced immunodevice was tested

by detecting other biomolecules such as PAMV and C-reactive protein (CRP).

4.2 Immunodevice fabrication and detection mechanism

The immunodevice is an analytical tool that allows for the detection of various biomarkers with high sensitivity and specificity. The biotin-streptavidin system, which is utilized in the immunodevice preparation, is a highly versatile and effective approach for immobilizing biomolecules and amplifying signals. The immobilization of biomolecules on the electrode surface through the biotin-streptavidin system offers stable signal amplification, selectivity, and specificity, making it an ideal choice for the fabrication of immunodevices. The three steps involved in the immunodevice fabrication ensure that the biomolecules are properly immobilized on the electrode surface and are available for electrochemical detection. The first step in the fabrication of the immunodevice involves modifying the SPCE to make it suitable for immobilizing the biomolecules. This is achieved by coating the SPCE surface with a layer of suitable material that allows for stable immobilization of the biomolecules. In the second step, the biomolecules are immobilized on the modified SPCE using the biotin-streptavidin system. Finally, in the third step, the immunocomplex formed by the binding of the immobilized biomolecules with their corresponding target biomolecules is detected using electrochemical methods.

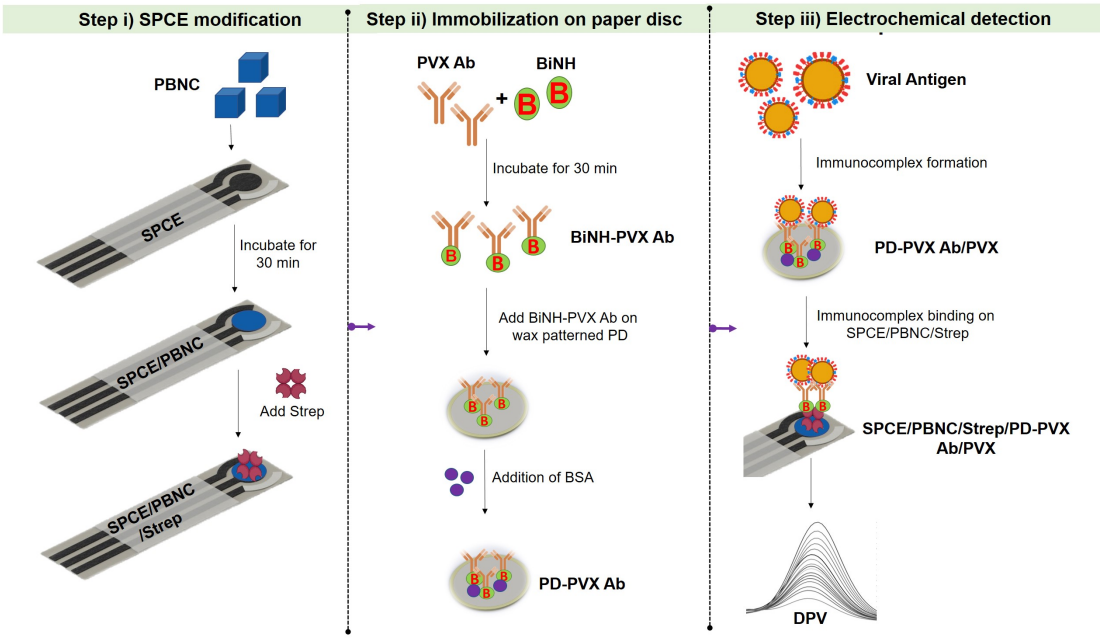


Figure 4.1: The mechanism of analysis and detection principles utilized in an immunodevice for detecting PVX

4.3 Analytical performance of the immunodevice

The analytical performance of the developed immunodevice was investigated with different PVX concentrations using DPV under optimal conditions. The immunocomplex formation on the electrode was examined using DPV at a scan rate of 0.1 V/s with an Estep of 0.01 V and tpulse time value of 0.02 s. As depicted in **Figure 4.2a**, the current decreases as the PVX concentration increases within the range of 800000–0.008 μM. This decline in current is ascribed to hindered electron transfer due to the formation of an insulating layer on the electrode surface caused by increased antigen binding. The calibration curve was generated by plotting the current versus the PVX concentration, yielding a regression equation of y = -860.07x + 279.11, R^2 = 0.97, with a low detection limit (LOD = 3S/N) of 0.92 nM (0.00092 μM) **(Figure 4.2b)**.

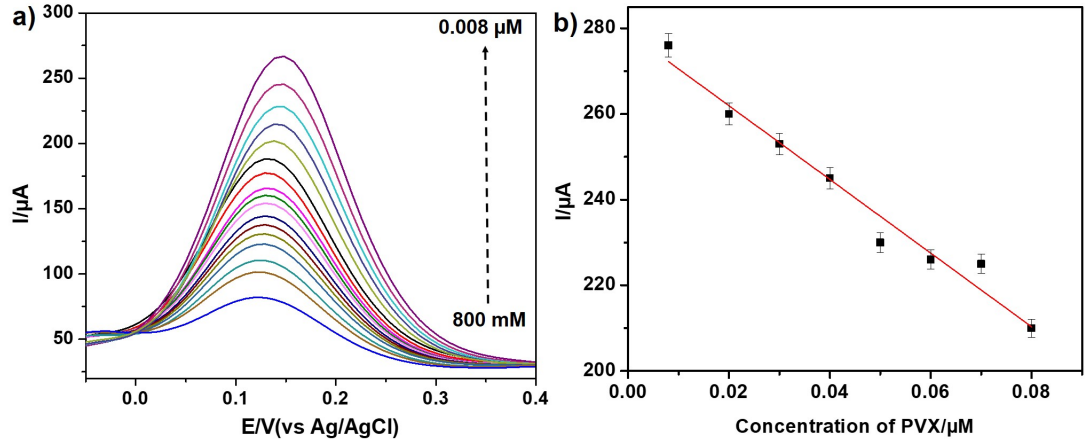


Figure 4.2: Differential pulse voltammetry (DPV) response of: a) The immunodevice for various standard concentrations of PVX antigen (800000, 800, 80, 8, 0.8, 0.72, 0.56, 0.40, 0.24, 0.08, 0.07, 0.05, 0.04, 0.02, and 0.008 μM) b) The calibration curve of the current response as a function of PVX antigen concentration is also shown, with mean ± S. E values (R^2 = 0.97, slope = -860.07, and intercept = 279.11).

4.4 Validation of the immunodevice performance in real samples

The effectiveness of the PVX immunodevice was further evaluated by testing it on actual potato leaf and tuber samples collected from a farm with minimal preprocessing. The samples were manually homogenized and extracted using a mortar and pestle, and then analyzed using the immunodevice in triplicate **(Figure ?? a)**. An image of the PD, foldable plastic housing, and PD-interfaced immunodevice for PVX detection is provided in **Figure ?? b**. A total of 20 samples were collected from Ghanshyam farms in Gujarat,

India, with 10 samples each for potato leaves and tubers. These samples underwent rigorous validation, with statistical parameters such as standard deviation, coefficient of variance, and relative error determined for each sample. The concentration of PVX in the plant supernatant was determined using the immunodevice and compared to a commercial ELISA test ($p < 0.05$).

The study involved analyzing ten samples of potato leaves using an immunodevice. The results of the DPV analysis, conducted in triplicate, are presented in **Figure 4.3**.

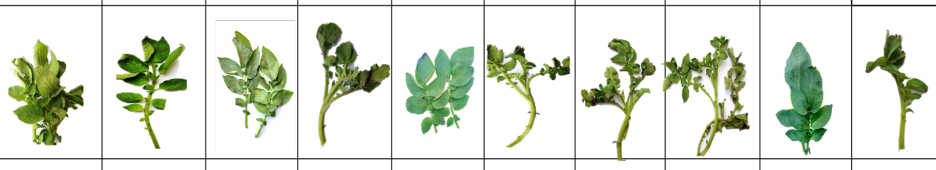
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Sample										
Estimated concentration	7.885 mM	0.922 µM	0.082 µM	0.825 µM	0.038 mM	0.020 µM	0.707 µM	0.721 µM	0.244 µM	0.019 µM
Standard deviation	5	5.29	6.50	6.49	10.14	12.50	4.04	10	7.02	13.65
Relative error	2.88	3.05	3.75	3.71	5.85	7.21	2.33	5.77	4.05	7.88
Coefficient of variance	4.27	3.26	3.68	4.50	4.53	5.15	2.51	6.49	3.88	5.55

Figure 4.3: Photographs showing potato leaf samples used for the immunodevice validation along with estimated concentration and statistical data

These results were then compared with the DPV values for PVX to establish the detection threshold, which is shown in **Figure 4.4**. The lowest concentration from the DPV analysis of standard PVX was considered as the threshold, and all analyzed samples were found to be within this range, as shown in **Figure 4.5a**.

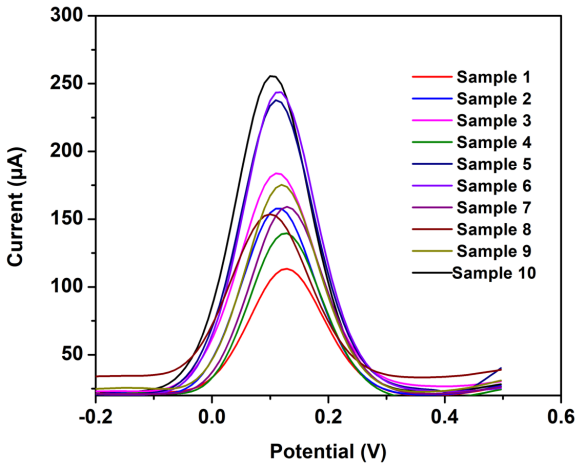


Figure 4.4: DPV curves showing the response of immunodevice towards the potato leaf extracts from the respective potato leaf samples alongside standard PVX reading as a threshold

The concentration of PVX was estimated for each analyzed sample using the standard curve, and this is shown in **Figure 4.5b** in comparison to the estimated concentra-

tion from an ELISA test. The ELISA test detected PVX in only four samples, while the immunodevice detected PVX in all ten samples, indicating its high sensitivity compared to the ELISA system.

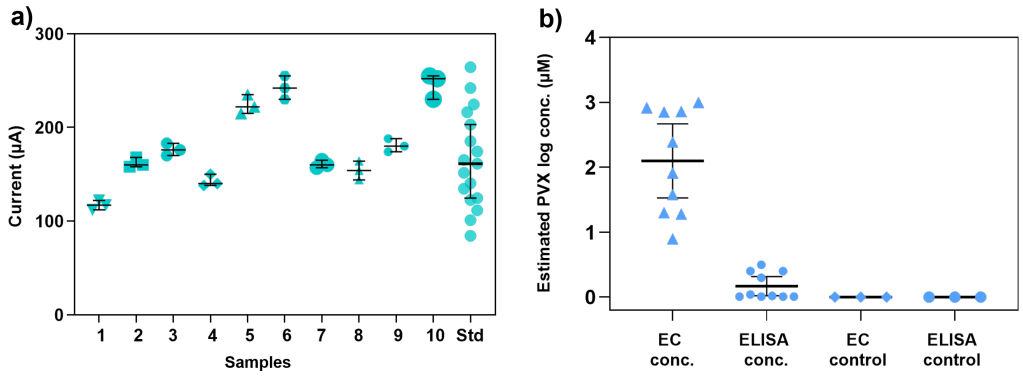


Figure 4.5: a) Plot of the current response as a function of concentration of PVX in potato leaf samples (n = 3, mean ± S. E); b) plot of estimated concentration of immunodevice and ELISA test for PVX in potato leaf samples

The study also involved analyzing ten potato tuber samples using the immunodevice. The tuber samples were analyzed in triplicate and compared to the standard DPV values for PVX to establish the detection threshold, as shown in **Figure 4.6 and 4.7** .

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Sample										
Estimated concentration	0.05 µM	0.042 µM	0.018 µM	7.88 µM	81.22 µM	87.67 mM	0.807 µM	0.079 µM	0.07 µM	0.06 µM
Standard deviation	23.35	8.54	17.03	15.17	15.41	7.93	12.89	24.0	10.53	14.73
Relative error	13.48	4.93	9.83	8.76	8.89	4.58	7.44	13.86	6.08	8.50
Coefficient of variance	6.39	3.69	6.56	11.13	10.96	10.44	9.87	9.32	4.99	4.05

Figure 4.6: Photographs showing potato tuber samples used for the immunodevice validation along with estimated concentration and statistical data

Samples 1 and 10 were found to have concentrations above the threshold, while the remaining samples were found to be within the threshold range, this could be attributed to the complexity of plant samples and variations in the electrode surface. The immunodevice showed significantly different currents from the negative control for all 20 samples ($p < 0.05$) displaying its ability to differentiate between positive and negative samples.

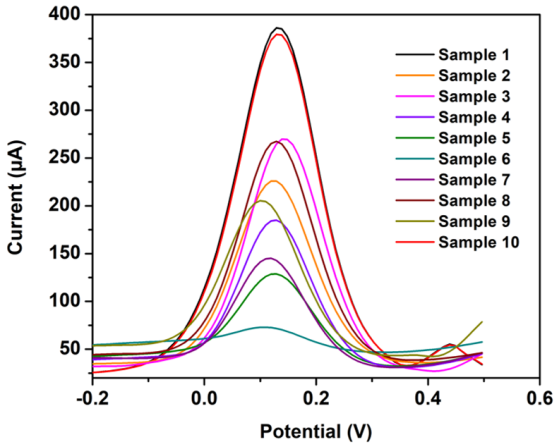


Figure 4.7: DPV curves showing the response of immunodevice towards the potato tuber extracts from the respective potato tuber samples alongside standard PVX reading as a threshold

The PVX concentration was estimated for each analyzed sample using the standard curve and compared to the estimated concentration from an ELISA test. The ELISA test results displayed positive results in one sample only for PVX detection. However, the immunodevice was able to detect PVX in eight samples, with two samples exhibiting current values beyond the detection range of the immunodevice **(Figure 4.8a and b).**

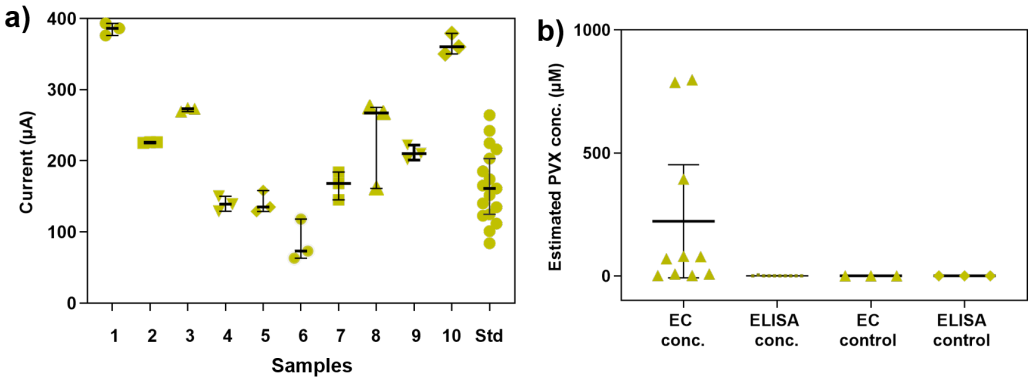


Figure 4.8: a) Plot of the current response as a function of concentration of PVX in potato tuber samples (n = 3, mean ± S. E); b) plot of estimated concentration of immunodevice and ELISA test for PVX in potato tuber samples

These results demonstrate the immunodevice ability to discriminate between negative and positive samples and detect even trace levels of PVX that are undetectable using ELISA. Therefore, the immunodevice has the potential to accurately quantify PVX antigen in real samples.

4.5 Extension of the immunodevice platform for the detection of diverse biomarkers

The versatility of the immunodevice platform was further demonstrated by targeting other biomarkers and recording their electrochemical responses. The platform utilized a replaceable universal detector electrode (SPCE/PBNC/Strep) and biotin-streptavidin detection to recognize PAMV and CRP biomarkers. The electrochemical responses to various concentrations of PAMV and CRP were recorded, as shown in **Figure 4.9 a, b**. A noticeable decrease in DPV current was observed with an increase in biomarker concentration for both PAMV and CRP antigens. The resulting calibration curves showed that the platform could directly detect concentrations ranging from 0.0001 to 1μM for PAMV and from 1 to 0.01 mg mL^{1} for CRP. The correlation equations were y = 82.98 + (-2.993) ×, R^2 = 0.955 for PAMV detection and y = 124.6 + (-3338.04) ×, R^2 = 0.97 for CRP detection, as shown in Figure S6 c and d, respectively. These results indicate the potential of the immunodevice platform to detect a wide range of biomarkers with high sensitivity and accuracy.

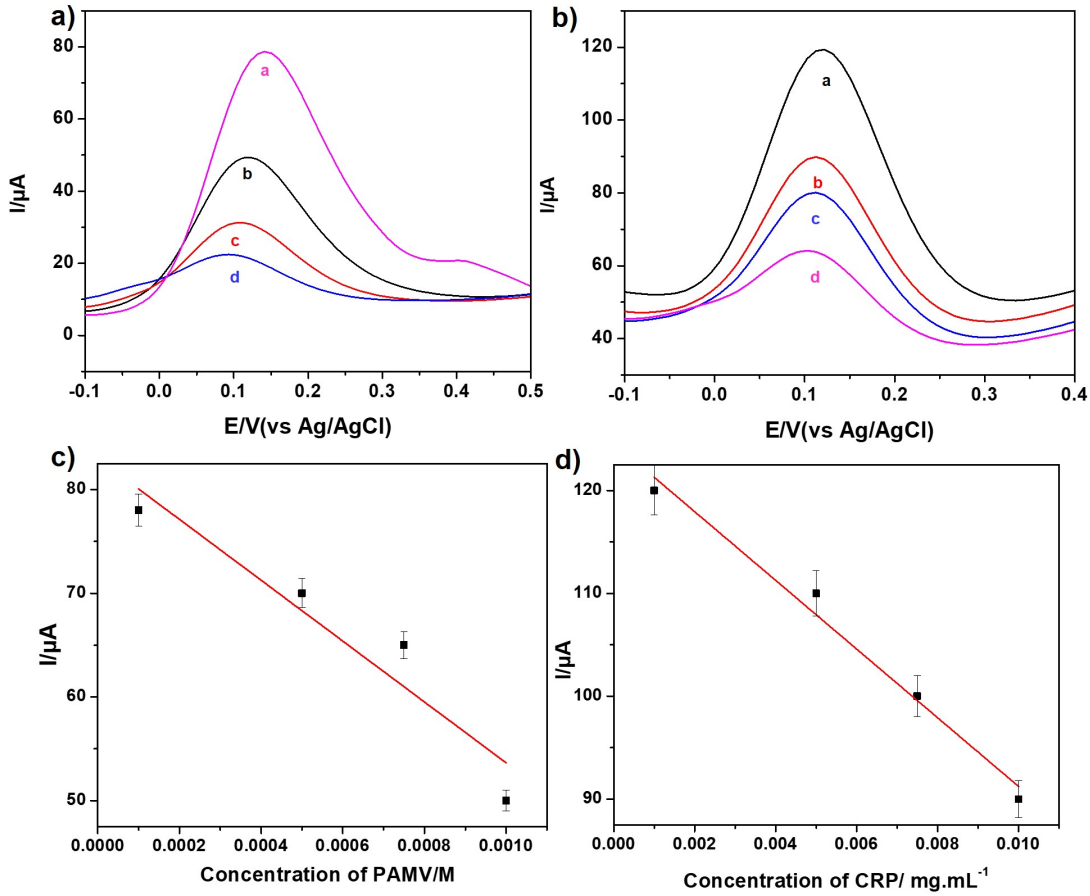


Figure 4.9: a) DPV graph toward different concentrations of standard PAMV (0.1 - 0.001 M) b) Calibration curve of the current response as a function of concentration of PAMV antigen c) DPV graph toward different concentrations of standard C-reactive protein (1 - 0.01 mg/ml) d) Calibration curve of the current response as a function of concentration of C-reactive protein

The immunodevice developed in this study has the potential to facilitate early detection of biomarkers and enable prompt diagnosis at the point of care. The device generates discriminatory signals in a matter of seconds to minutes, with minimal user interpretation required, thereby streamlining the diagnostic process. In terms of cost, the device utilizes printed electrodes, which are scalable and help minimize test expenses. Traditional protein detection technologies currently take 24 hours for sample analysis, but the immunodevice can significantly reduce assay time and provide while-you-wait results.

4.6 Conclusion

To facilitate the quick and easy detection of various biomarkers at the point of care (POC), a team of researchers developed a versatile immunodevice that utilizes a streptavidin-biotin detection strategy. The device includes a replaceable probe designed to target multiple biomarkers simultaneously, making it a valuable diagnostic tool for various medical applications. The immunodevice was able to detect PVX within a dynamic range of 800,000 to 0.008 μM, and it was found that it performed with high sensitivity when cross checked with close biomarkers. The immunodevice exhibited a limit of detection (LOD) of 0.92 nM. Furthermore, the immunodevice was able to detect real samples of potato leaves and tubers, demonstrating its potential as a POC diagnostic platform. The immunodevice also proved to be a universal sensing platform for detecting CRP and PAMV, thanks to its flexible architecture and recognition probe. The device satisfied the ASSURED standards, meaning it is a low-cost, highly sensitive, and selective tool. With its easy-to-use interface, providing quick analysis (2-3 min) through a smartphone-enabled operation, the immunodevice has great potential as a diagnostic tool in resource-limited settings. Overall, the device is a promising solution for the early detection of various biomarkers, enabling timely diagnosis and improving patient outcomes.

Contributions of the Thesis

1. Technical advancement over the existing products/technologies using effective bio-conjugtaion strategy
2. Detection of multiple biomarkers using a single device we have tested using plant biomarkers such as PAMV, and PVY and human biomarker like C-reactive protein
3. Development of TLR-3/4 level pythodigansotic kits for detection of PVY and PVX

Scope of Future Work

ven though we have attempted and developed diagnostics tools for the detection of viruses such as PVY, PAMV, and PVX, rigorous validation is required in order to push forward the TLR levels. Also, translating elelctrochemical sensor into paper strips could be the next progression in pythodiagnsotics. The exploration of a more effective detection strategy for application in biosensor is need for the hour. The non-invasive monitoring of plants using biosensors could provide valuable data for precision agriculture, optimizing resource management, and early detection of plant diseases.

List of Publications

- Alok Pandya and Nidhi Verma. A DIPSTRIP- VERTICAL FLOW-BASED IMMUNOASSAY FOR DETECTION OF PLANT PATHOGENS".202321006356 filed on 31/01/2023

Research article

- Verma, N. V., Tiwari, B. S., Pandya, A. (2023). Paper disc interfaced Prussian blue nanocube modified immunodevice for electrochemical detection of diverse biomarker at point of care. Bioelectrochemistry,
- Verma, N., Tiwari , B.S. and Pandya , A. (2022) "Development of PAMV viral electrochemical immunosensor exploring shape dependent nanomatrix immobilization strategy". Potato Research 150, 108346.
- Verma, N. et al. (2022) "Tailored calix[4]arene-gold nanoconjugate as a ultrasensitive immunosensing nanolabel," Biomedical Microdevices, 25(1).
- Vora, M. et al. (2022) "Oxacalix[4]arene based dual-signalling fluorimetric and electrochemical chemosensor for the selective detection of nitroaromatic compounds," Journal of Molecular Liquids, 362, p. 119791.
- Verma, N., Tiwari, B. S., Pandya, A. (2021). Field Deployable Vertical Flow Based Immunodevice for Detection of Potato Virus Y in Potato Leaves. ACS Agricultural Science Technology, 1(5), 558-565.
- Kongor, A., Panchal, M., Athar, M., Vora, M., Verma, N., Pandya, A., ... Jain, V. (2020). Colorimetric and electrochemical sensing of As (III) using calix [4] pyrrole capped gold nanoparticles and evaluation of its cytotoxic activity. Journal of Inclusion Phenomena and Macrocyclic Chemistry, 98(1), 29-41.
- Prajapati, A., Verma, N., Pandya, A. (2020). Highly sensitive vertical flow based point-of-care immunokit for rapid and early detection of human CRP as a cardiovascular risk factor. Biomedical Microdevices, 22(2), 28.

Review article

- Shukla, M.,Verma, N.V., et.al (2023).A Review on Tunable Multi-Functional Prussian Blue Nanoparticles; Their Promising Biological Applications Future Direc-

tions.Coordination Chemistry Reviews.[Under review:CCR-D-23-00628]

- Verma, N.V., Shukla, M., Kulkarni, R., Srivastava, K., Claudic, B., Savara, J., Joseph Mathew, M., Maurya, R., Bhattacharjee, G., Singh, V. and Pandya, A., (2022). Emerging Extraction and Diagnostic Tools for Detection of Plant Pathogens: Recent Trends, Challenges, and Future Scope.ACS Agricultural Science Technology.

- Verma, N., Patel, D. and Pandya, A. (2020) "Emerging diagnostic tools for detection of COVID-19 and perspective," Biomedical Microdevices, 22(4).

Chapter

- Verma, N., Walia, S. and Pandya, A. (2022) "Micro/nanofluidic devices for DNA/RNA detection and separation," Progress in Molecular Biology and Translational Science, pp. 85–107.

- Verma, N., Kulkarni, R. and Pandya, A. (2022) "Microfluidic tools for veterinary and Zoonotic Disease Diagnostics," Progress in Molecular Biology and Translational Science, pp. 281–293.

- Verma, N, Prajapati Parth, Singh Vijai, Pandya A, (2021).An introduction to microfluidics and their applications, Book on Micro/Nanofluidics and Lab-on-Chip Based Emerging Technologies for Biomedical and Translational Research Applications, Elsevier publisher.

- Verma, N,, Pandya A, (2021) Challenges and opportunities in micro/nanofluidic and lab-on-a-chip, Book on Micro/Nanofluidics and Lab-on-Chip Based Emerging Technologies for Biomedical and Translational Research Applications, Elsevier publisher.

- Verma, N. et al. (2020) "Antimicrobial nanomaterials for water disinfection," Nanotoxicity, pp. 365–383.

Conference Proceedings

Oral and poster presentations

- GTU Multi-displinary International conference, GTU ICON, 2022- (1st prize) (International Oral presentation)
- "C-DNA conference" – CeNSE DBT Nano-biotechnology alliance-IISc Bangalore, 2022 (International Oral presentation)
- IAR Research conclave, IAR, Gujarat, 2021 (National presentation)
- Eureka, IAR, Gujarat, 2020 (Poster presentation)

Workshops

- EMSL: Investigating and testing for healthcare associated infections, 2022
- "C-DNA familizaration"– CeNSE DBT Nnao-biotechnology alliance-IISc Bangalore-Level 1
- "C-DNA hands on"– CeNSE DBT Nano-biotechnology alliance-IISc Bangalore-Level 2
- "C-DNA" basic training on nanotechnology – CeNSE DBT Nano-biotechnology alliance-IISc Bangalore

References

[1] U. Nations, "Feeding the World Sustainably | United Nations — un.org," https://www.un.org/en/chronicle/article/feeding-world-sustainably, [Accessed 24-Jun-2023].

[2] "UN Report: Global hunger numbers rose to as many as 828 million in 2021 — fao.org," https://www.fao.org/newsroom/detail/un-report-global-hunger-SOFI-2022-FAO/en, [Accessed 24-Jun-2023].

[3] "FAO -xA0;News Article:xA0;2050: A third more mouths to feed — fao.org," https://www.fao.org/news/story/en/item/35571/icode/, [Accessed 24-Jun-2023].

[4] "Hunger Levels Continue on the Rise: 2022 Global Hunger Index Released | Africa South of the Sahara — ssa.foodsecurityportal.org," https://ssa.foodsecurityportal.org/node/2078, [Accessed 24-Jun-2023].

[5] N. V. Verma, M. Shukla, R. Kulkarni, K. Srivastava, B. Claudic, J. Savara, M. Joseph Mathew, R. Maurya, G. Bhattacharjee, V. Singh, and A. Pandya, "Emerging extraction and diagnostic tools for detection of plant pathogens: Recent trends, challenges, and future scope," *ACS Agric. Sci. Technol.*, vol. 2, no. 5, pp. 858–881, Oct. 2022.

[6] "Card zh — fao.org," https://www.fao.org/documents/card/zh/c/cc3751en/, 2017, [Accessed 24-Jun-2023].

[7] "FAOSTAT — faostat.fao.org," http://faostat.fao.org/, [Accessed 24-Jun-2023].

[8] R. Rana and P. Govindakrishnan, *Vision 2050, Central Potato Research Insitute, Shimla*, 01 2014.

[9] M. Singh, K. Mukherjee, S. Khurana, G. Jai, M. Querci *et al.*, "Detection of potato spindle tuber viroid by nash in exotic potato germplasm." in *Potato, global research & development. Proceedings of the Global Conference on Potato, New Delhi, India, 6-11 December, 1999: Volume 1*. Indian Potato Association, 2000, pp. 491–494.

[10] S. P. Khurana and M. Singh, "Yield loss potential of potato viruses x and y in indian potatoes," *J Indian Potato Assoc*, vol. 15, pp. 27–29, 1988.

[11] I. Garg, S. P. Khurana, K. Shiv, B. Lakra *et al.*, "Association of a geminivirus with potato apical leaf curl in india and its immuno-electron microscopic detection." *Journal of the Indian Potato Association*, vol. 28, no. 2/4, pp. 227–232, 2001.

[12] B. Lakra, "Potato apical leaf curl begomovirus-symptoms, appraisal of a scale and losses in potato crop," *Journal of Indian Potato Association*, vol. 30, pp. 119–120, 2003.

[13] R. N. Salaman and W. G. Burton, *The history and social influence of the potato*. Cambridge University Press, 1985.

[14] L. F. Salazar, *Potato viruses and their control*. International Potato Center, 1996.

[15] O. Appel, *Neuere Untersuchungen Über Kartoffel-Und Tomaten-Erkrankungen*. Borntraeger, 1906.

[16] H. M. Quanjer, *Nature, Mode of Dissemination and Control of Phloem-necrosis Leaf-roll and Related Diseases IA Sereh*, 1916.

[17] E. Schultz, "Transmission, variation, and control of cer," *Journal of Agricultural Research*, vol. 25, p. 43, 1923.

[18] D. Van Slogteren, "Preparation of antisera against potato virus s with special reference to the preparation of non-toxic virus-suspensions for the immunization of rabbits from extracts of infected potato plants," in *Proc Conf on Potato Virus Dis, 2nd. Lisse-Wageningen, Netherlands*, 1955, pp. 35–42.

[19] S. R and F. C. Mellor, "Eradication of potato viruses x and s by thermotherapy and axillary bud culture," *Phytopathology*, vol. 58, no. 2, p. 199, 1968.

[20] G. Loebenstein and V. Gaba, "Viruses of potato," in *Advances in virus research*. Elsevier, 2012, vol. 84, pp. 209–246.

[21] F. Legorburu, R. Marquínez, and J. Ruiz de Gauna, "Spatiotemporal gradients of pvy infection from an external source," in *Abstr 13th Triennial Conf. EAPR, Veldhoven, The Netherlands*, 1996, pp. 120–121.

[22] D. Lo Presti, J. Di Tocco, C. Massaroni, S. Cimini, L. De Gara, S. Singh, A. Raucci, G. Manganiello, S. L. Woo, E. Schena, and S. Cinti, "Current understanding, challenges and perspective on portable systems applied to plant monitoring and precision agriculture," *Biosensors and Bioelectronics*, vol. 222, p. 115005, 2023. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S0956566322010454

[23] A. Blanchard, M. Rolland, C. Lacroix, C. Kerlan, and E. Jacquot, "Potato virus y: a century of evolution," *Virology*, vol. 7, pp. 21–32, 2008.

[24] Y. F. Drygin, A. N. Blintsov, V. G. Grigorenko, I. P. Andreeva, A. P. Osipov, Y. A. Varitzev, A. I. Uskov, D. V. Kravchenko, and J. G. Atabekov, "Highly sensitive field test lateral flow immunodiagnostics of pvx infection," *Applied microbiology and biotechnology*, vol. 93, pp. 179–189, 2012.

[25] S. Hill and E. A. Jackson, "An investigation of the reliability of elisa as a practical test for detecting potato leaf roll virus and potato virus y in tubers," *Plant Pathology*, vol. 33, no. 1, pp. 21–26, 1984.

[26] R. P. Singh and M. Singh, "Specific detection of potato virus a in dormant tubers by reverse-transcription polymerase chain reaction," *Plant disease*, vol. 82, no. 2, pp. 230–234, 1998.

[27] R. P. Singh, "Reverse-transcription polymerase chain reaction for the detection of viruses from plants and aphids," *Journal of Virological methods*, vol. 74, no. 2, pp. 125–138, 1998.

[28] J. H. Lorenzen, L. M. Piche, N. C. Gudmestad, T. Meacham, and P. Shiel, "A multiplex pcr assay to characterize potato virus y isolates and identify strain mixtures," *Plant Disease*, vol. 90, no. 7, pp. 935–940, 2006.

[29] M. Singh, R. P. Singh, M. Fageria, X. Nie, R. Coffin, and G. Hawkins, "Optimization of a real-time rt-pcr assay and its comparison with elisa, conventional rt-pcr and the grow-out test for large scale diagnosis of potato virus y in dormant potato tubers," *American Journal of Potato Research*, vol. 90, pp. 43–50, 2013.

[30] X. Nie, "Reverse transcription loop-mediated isothermal amplification of dna for detection of potato virus y," *Plant Disease*, vol. 89, no. 6, pp. 605–610, 2005.

[31] M. Almasi, S. Dehabadi *et al.*, "Colorimetric immunocapture reverse transcription loop-mediated isothermal amplification assay for rapid detection of the potato virus y," *J. Plant Pathol. Microb*, vol. 4, p. 188, 2013.

[32] M. M. López, P. Llop, A. Olmos, E. Marco-Noales, M. Cambra, and E. Bertolini, "Are molecular tools solving the challenges posed by detection of plant pathogenic bacteria and viruses?" *Current issues in molecular biology*, vol. 11, no. 1, pp. 13–46, 2009.

[33] S. Souf, "Recent advances in diagnostic testing for viral infections. biosci horizons int," *J. Stud. Res*, vol. 9, 2016.

[34] S. Pillai, *Rapid molecular detection of microbial pathogens: breakthroughs and challenges*. Springer, 1997.

[35] A. W. Martinez, S. T. Phillips, G. M. Whitesides, and E. Carrilho, "Diagnostics for the developing world: microfluidic paper-based analytical devices," 2010.

[36] J. Wei, H. Liu, F. Liu, M. Zhu, X. Zhou, and D. Xing, "Miniaturized paper-based gene sensor for rapid and sensitive identification of contagious plant virus," *ACS applied materials & interfaces*, vol. 6, no. 24, pp. 22 577–22 584, 2014.

[37] C. Danks and I. Barker, "On-site detection of plant pathogens using lateral-flow devices," *EPPO Bulletin*, vol. 30, no. 3-4, pp. 421–426, 2000.

[38] M. Sajid, A.-N. Kawde, and M. Daud, "Designs, formats and applications of lateral flow assay: A literature review," *Journal of Saudi Chemical Society*, vol. 19, no. 6, pp. 689–705, 2015.

[39] J. D. Bishop, H. V. Hsieh, D. J. Gasperino, and B. H. Weigl, "Sensitivity enhancement in lateral flow assays: A systems perspective," *Lab on a Chip*, vol. 19, no. 15, pp. 2486–2499, 2019.

[40] T. Mahmoudi, M. de la Guardia, B. Shirdel, A. Mokhtarzadeh, and B. Baradaran, "Recent advancements in structural improvements of lateral flow assays towards point-of-care testing," *TrAC Trends in Analytical Chemistry*, vol. 116, pp. 13–30, 2019.

[41] N. Jiang, R. Ahmed, M. Damayantharan, B. Ünal, H. Butt, and A. K. Yetisen, "Lateral and vertical flow assays for point-of-care diagnostics," *Advanced healthcare materials*, vol. 8, no. 14, p. 1900244, 2019.

[42] G. M. Ross, G. I. Salentijn, and M. W. Nielen, "A critical comparison between flow-through and lateral flow immunoassay formats for visual and smartphone-based multiplex allergen detection," *Biosensors*, vol. 9, no. 4, p. 143, 2019.

[43] A. Pandya, P. G. Sutariya, A. Lodha, and S. K. Menon, "A novel calix [4] arene thiol functionalized silver nanoprobe for selective recognition of ferric ion with nanomolar sensitivity via dls selectivity in human biological fluid," *Nanoscale*, vol. 5, no. 6, pp. 2364–2371, 2013.

[44] A. Pandya, P. G. Sutariya, and S. K. Menon, "A non enzymatic glucose biosensor based on an ultrasensitive calix [4] arene functionalized boronic acid gold nanoprobe for sensing in human blood serum," *Analyst*, vol. 138, no. 8, pp. 2483–2490, 2013.

[45] N. Verma, B. S. Tiwari, and A. Pandya, "Field deployable vertical flow based immunodevice for detection of potato virus Y in potato leaves," *ACS Agric. Sci. Technol.*, vol. 1, no. 5, pp. 558–565, Oct. 2021.

[46] J. F. Kreuze, J. Souza-Dias, A. Jeevalatha, A. Figueira, J. Valkonen, and R. Jones, "Viral diseases in potato," *The potato crop: its agricultural, nutritional and social contribution to humankind*, pp. 389–430, 2020.

[47] R. G. Samson, T. C. Allen, and J. L. Whitworth, "Evaluation of direct tissue blotting to detect potato viruses," *American Potato Journal*, vol. 70, pp. 257–265, 1993.

[48] H. Xu, D. Leclerc, B. Leung, and M. AbouHaidar, "The entire nucleotide sequence and genomic organization of potato aucuba mosaic potexvirus," *Archives of virology*, vol. 135, pp. 461–469, 1994.

[49] R. Koenig and H. Paul, "Variants of elisa in plant virus diagnosis," *Journal of Virological Methods*, vol. 5, no. 2, pp. 113–125, 1982.

[50] B. O. Agindotan, P. J. Shiel, and P. H. Berger, "Simultaneous detection of potato viruses, plrv, pva, pvx and pvy from dormant potato tubers by taqman® real-time rt-pcr," *Journal of virological methods*, vol. 142, no. 1-2, pp. 1–9, 2007.

[51] T. Maoka, S. Sugiyama, Y. Maruta, and T. Hataya, "Application of cdna macroarray for simultaneous detection of 12 potato viruses," *Plant Disease*, vol. 94, no. 10, pp. 1248–1254, 2010.

[52] K. Omidfar, F. Khorsand, and M. D. Azizi, "New analytical applications of gold nanoparticles as label in antibody based sensors," *Biosensors and Bioelectronics*, vol. 43, pp. 336–347, 2013.

[53] O. A. Sadik, A. O. Aluoch, and A. Zhou, "Status of biomolecular recognition using electrochemical techniques," *Biosensors and Bioelectronics*, vol. 24, no. 9, pp. 2749–2765, 2009.

[54] B. Cai, S. Wang, L. Huang, Y. Ning, Z. Zhang, and G.-J. Zhang, "Ultrasensitive label-free detection of pna–dna hybridization by reduced graphene oxide field-effect transistor biosensor," *ACS nano*, vol. 8, no. 3, pp. 2632–2638, 2014.

[55] W.-M. Munief, X. Lu, T. Teucke, J. Wilhelm, A. Britz, F. Hempel, R. Lanche, M. Schwartz, J. K. Y. Law, S. Grandthyll *et al.*, "Reduced graphene oxide biosensor platform for the detection of nt-probnp biomarker in its clinical range," *Biosensors and Bioelectronics*, vol. 126, pp. 136–142, 2019.

[56] P. Yanez-Sedeno and J. Pingarron, "Gold nanoparticle-based electrochemical biosensors," *Analytical and bioanalytical chemistry*, vol. 382, pp. 884–886, 2005.

[57] S. I. Kaya, L. Karadurmus, G. Ozcelikay, N. K. Bakirhan, and S. A. Ozkan, "Electrochemical virus detections with nanobiosensors," in *Nanosensors for smart cities*. Elsevier, 2020, pp. 303–326.

[58] M. Sharafeldin, K. McCaffrey, and J. F. Rusling, "Influence of antibody immobilization strategy on carbon electrode immunoarrays," *Analyst*, vol. 144, no. 17, pp. 5108–5116, 2019.

[59] V. Naresh and N. Lee, "A review on biosensors and recent development of nanostructured materials-enabled biosensors," *Sensors*, vol. 21, no. 4, p. 1109, 2021.

[60] W. Jin, G. Wu, and A. Chen, "Sensitive and selective electrochemical detection of chromium (vi) based on gold nanoparticle-decorated titania nanotube arrays," *Analyst*, vol. 139, no. 1, pp. 235–241, 2014.

[61] B. Saha, T. H. Evers, and M. W. Prins, "How antibody surface coverage on nanoparticles determines the activity and kinetics of antigen capturing for biosensing," *Analytical chemistry*, vol. 86, no. 16, pp. 8158–8166, 2014.

[62] J. E. Lee, J. H. Seo, C. S. Kim, Y. Kwon, J. H. Ha, S. S. Choi, and H. J. Cha, "A comparative study on antibody immobilization strategies onto solid surface," *Korean Journal of Chemical Engineering*, vol. 30, pp. 1934–1938, 2013.

[63] J. N. Tiwari, V. Vij, K. C. Kemp, and K. S. Kim, "Engineered carbon-nanomaterial-based electrochemical sensors for biomolecules," *ACS nano*, vol. 10, no. 1, pp. 46–80, 2016.

[64] C. M. Pandey, S. Augustine, S. Kumar, S. Kumar, S. Nara, S. Srivastava, and B. D. Malhotra, "Microfluidics based point-of-care diagnostics," *Biotechnology journal*, vol. 13, no. 1, p. 1700047, 2018.

[65] L. Sutarlie, D. Siak-Wie Ow, S. Kong Ng, Y. Yang, and X. Su, "Gold nanoparticle-based "mix and measure" fluorimetric assays to quantify antibody titer," *Chemistry–An Asian Journal*, vol. 16, no. 20, pp. 3188–3193, 2021.

[66] F. Du, Y. Chen, C. Meng, B. Lou, W. Zhang, and G. Xu, "Recent advances in electrochemiluminescence immunoassay based on multiple-signal strategy," *Current Opinion in Electrochemistry*, vol. 28, p. 100725, 2021.

[67] J. Yang, K. Wang, H. Xu, W. Yan, Q. Jin, and D. Cui, "Detection platforms for point-of-care testing based on colorimetric, luminescent and magnetic assays: A review," *Talanta*, vol. 202, pp. 96–110, 2019.

[68] J. Y. Lichtenberg, Y. Ling, and S. Kim, "Non-specific adsorption reduction methods in biosensing," *Sensors*, vol. 19, no. 11, p. 2488, 2019.

[69] J. E. Contreras-Naranjo and O. Aguilar, "Suppressing non-specific binding of proteins onto electrode surfaces in the development of electrochemical immunosensors," *Biosensors*, vol. 9, no. 1, p. 15, 2019.

[70] Y.-H. Chen, W.-C. Chien, D.-C. Lee, and K.-T. Tan, "Signal amplification and detection of small molecules via the activation of streptavidin and biotin recognition," *Analytical chemistry*, vol. 91, no. 19, pp. 12 461–12 467, 2019.

[71] C. M. Dundas, D. Demonte, and S. Park, "Streptavidin–biotin technology: improvements and innovations in chemical and biological applications," *Applied microbiology and biotechnology*, vol. 97, pp. 9343–9353, 2013.

[72] L. Chaiet and F. J. Wolf, "The properties of streptavidin, a biotin-binding protein produced by streptomycetes," *Archives of biochemistry and biophysics*, vol. 106, pp. 1–5, 1964.

[73] P. S. Stayton, S. Freitag, L. A. Klumb, A. Chilkoti, V. Chu, J. E. Penzotti, R. To, D. Hyre, I. Le Trong, T. P. Lybrand *et al.*, "Streptavidin–biotin binding energetics," *Biomolecular engineering*, vol. 16, no. 1-4, pp. 39–44, 1999.

[74] Y. Wang, G. Zhao, H. Wang, Y. Zhang, N. Zhang, D. Wei, R. Feng, and Q. Wei, "Label-free electrochemical immunosensor based on biocompatible nanoporous fe 3 o 4 and biotin–streptavidin system for sensitive detection of zearalenone," *Analyst*, vol. 145, no. 4, pp. 1368–1375, 2020.

[75] K. Zhang, G. Liu, and E. M. Goldys, "Robust immunosensing system based on biotin-streptavidin coupling for spatially localized femtogram ml- 1 level detection of interleukin-6," *Biosensors and Bioelectronics*, vol. 102, pp. 80–86, 2018.

[76] X. Liu, Y. Wen, W. Wang, Z. Zhao, Y. Han, K. Tang, and D. Wang, "Nanobody-based electrochemical competitive immunosensor for the detection of afb 1 through afb 1-hcr as signal amplifier," *Microchimica Acta*, vol. 187, pp. 1–10, 2020.

[77] N. Sandhyarani, "Surface modification methods for electrochemical biosensors," in *Electrochemical Biosensors*. Elsevier, 2019, pp. 45–75.

[78] B. Haghighi, H. Hamidi, and L. Gorton, "Electrochemical behavior and application of prussian blue nanoparticle modified graphite electrode," *Sensors and Actuators B: Chemical*, vol. 147, no. 1, pp. 270–276, 2010.

[79] S. Cinti, R. Cusenza, D. Moscone, and F. Arduini, "based synthesis of prussian blue nanoparticles for the development of whole blood glucose electrochemical biosensor," *Talanta*, vol. 187, pp. 59–64, 2018.

[80] P. C. Pandey, S. Singh, and S. N. Sawant, "Functional alkoxysilane mediated controlled synthesis of prussian blue nanoparticles, enabling silica alginate bead development; nanomaterial for selective electrochemical sensing," *Electrochimica Acta*, vol. 287, pp. 37–48, 2018.